

This Page Is Inserted by IFW Operations
and is not a part of the Official Record

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.


**As rescanning documents *will not* correct images,
please do not report the images to the
Image Problem Mailbox.**

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference P9803	FOR FURTHER ACTION		See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
International application No. PCT/NO99/00266	International filing date (<i>day/month/year</i>) 25/08/1999	Priority date (<i>day/month/year</i>) 26/08/1998	
International Patent Classification (IPC) or national classification and IPC A61K9/16			
Applicant NORSK HYDRO ASA et al			
<p>1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.</p> <p>2. This REPORT consists of a total of 8 sheets, including this cover sheet.</p> <p><input checked="" type="checkbox"/> This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).</p> <p>These annexes consist of a total of 2 sheets.</p>			
<p>3. This report contains indications relating to the following items:</p> <ul style="list-style-type: none"> I <input checked="" type="checkbox"/> Basis of the report II <input type="checkbox"/> Priority III <input type="checkbox"/> Non-establishment of opinion with regard to novelty, inventive step and industrial applicability IV <input checked="" type="checkbox"/> Lack of unity of invention V <input checked="" type="checkbox"/> Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement VI <input type="checkbox"/> Certain documents cited VII <input type="checkbox"/> Certain defects in the international application VIII <input checked="" type="checkbox"/> Certain observations on the international application 			
Date of submission of the demand 18/03/2000		Date of completion of this report 27.12.2000	
Name and mailing address of the international preliminary examining authority:  European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465		Authorized officer Muller, I Telephone No. +49 89 2399 8716	



INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/NO99/00266

I. Basis of the report

1. This report has been drawn on the basis of *(substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments (Rules 70.16 and 70.17).)*:

Description, pages:

1-44 as originally filed

Claims, No.:

1-11 with telefax of 21/09/2000

Drawings, sheets:

1/7-7/7 as originally filed

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language: , which is:

- ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
- ☐ the language of publication of the international application (under Rule 48.3(b)).
- ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- ☐ contained in the international application in written form.
- ☐ filed together with the international application in computer readable form.
- ☐ furnished subsequently to this Authority in written form.
- ☐ furnished subsequently to this Authority in computer readable form.
- ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
- ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

- ☐ the description, pages:
- ☐ the claims, Nos.:

**INTERNATIONAL PRELIMINARY
EXAMINATION REPORT**

International application No. PCT/NO99/00266

☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

(Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

IV. Lack of unity of invention

1. In response to the invitation to restrict or pay additional fees the applicant has:

- ☐ restricted the claims.
☐ paid additional fees.
☐ paid additional fees under protest.
☐ neither restricted nor paid additional fees.

2. ☒ This Authority found that the requirement of unity of invention is not complied and chose, according to Rule 68.1, not to invite the applicant to restrict or pay additional fees.

3. This Authority considers that the requirement of unity of invention in accordance with Rules 13.1, 13.2 and 13.3 is

- ☐ complied with.
☒ not complied with for the following reasons:
see separate sheet

4. Consequently, the following parts of the international application were the subject of international preliminary examination in establishing this report:

- ☒ all parts.
☐ the parts relating to claims Nos. .

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes: Claims 2-5,9-11
	No: Claims 1,6-8
Inventive step (IS)	Yes: Claims 2-5
	No: Claims 1,6-11

**INTERNATIONAL PRELIMINARY
EXAMINATION REPORT**

International application No. PCT/NO99/00266

Industrial applicability (IA) Yes: Claims 1-11
 No: Claims

2. Citations and explanations
see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:
see separate sheet

**INTERNATIONAL PRELIMINARY
EXAMINATION REPORT - SEPARATE SHEET**

International application No. PCT/NO99/00266

Re Item I

The disclaimer introduced in the sole independent claim 1 filed with letter of 21.11.00 received on 22.12.00 is not acceptable as D1, relating to the same technical field, cannot be considered as accidentally novelty destroying (Art. 19(2) PCT). Moreover, the fact that apoptosis of tumour cells is not explicitly referred to within the application documents does not exclude such apoptosis from the subject-matter of the application.

As a result this international preliminary examination report is drawn on the basis of the amendments filed by the applicant via telefax on 21.09.00.

Re Item IV

The subject-matter of independent claim 1 is already known (cf. item V). The requisite unity of invention (Rule 13.1 PCT) therefore no longer exists inasmuch as a technical relationship involving one or more of the same or corresponding special technical features in the sense of Rule 13.2 PCT does not exist between the subject-matter of the alternatives b) and d) defined in present claim 1 (Rule 13.3 PCT).

Re Item V

1. Reference is made to the following documents:

D1: WO-A1-9738707

D2: WO-A1-9628029

D3: Dong X. Yin, Li Zhu, R.T. Schimke, Tetracycline-Controlled Gene Expression System Achieves High-Level and Quantitative Control of Gene Expression. Analytical Biochemistry 1996, Vol. 235, No 0112, Pages 195-201.

D1 discloses devices for delivery of a cellular source of an apoptosis- inducing molecule (cf. claim 1 and FasL at. page 2, line 23 - page 3, line 11 and page 5, line 18 - page 6, line 12). The preferred devices used for the encapsulation of the cells have to fulfill the requirement of immunoisolation (p. 14, l. 29-30; p. 15, l. 1-6 and p. 16, l. 10-12) and may consist of alginate (p. 15, l. 9-14 and 29-30). At page 19, l. 10-11 reference is made to the treatment of brain tumours by said encapsulated source of FasL.

D2 discloses the immunoisolation of cells expressing therapeutic substances in composite microreactors (microcapsules) (p. 2, l. 3-5). One of the preferred

encapsulation matrices is alginate (p. 3, l. 13-14), the microreactors are in form of microbeads or microspheres (p. 65, l. 10-12). In this document moreover implantation of the beads into the brain (p. 74, l. 9-10) is envisaged, beads providing alpha-interferon are described at p. 74, l. 13-15 and treatment of several cancer forms (solid tumours, hair cell leukemia, Kaposi's sarcoma etc..) by microcapsules containing cells expressing alpha-interferon is described (p. 84, l. 21-33). No reference is made to using in alginate immunoisolated producer cells for treating specifically CNS tumours. In order to achieve effective immunoisolation properties, an outer layer is required to prevent cracking and subsequent host cell penetration through the encapsulation matrix (ex. 1, p. 75, l. 12-13).

2. Novelty (Article 33(2) PCT)

Claim 1 defines the use of a cell in general. It is not clear from the formulation of the claim that this cell is identical to the producer cell comprised in the defined medicament. Notwithstanding in the following, said cell is understood as the 'producer cell' defined by the applicant.

2.1 The wording "inhibition of tumour growth" in its broad meaning includes both slight slow down of tumour growth and prevention of tumour growth. The latter may consist for example in induced apoptosis of the tumour cells.

Moreover can the effect of the FasL protein on CNS tumour be classified under interaction with tumour/host communication pathways, said cell apoptosis influencing in some way these communication pathways. The same is applicable for the interference with the relationship of the tumour cell with the extracellular matrix. Thus, present claim 1 does not define any technical features providing distinction from the delivery device of producer cells expressing FasL protein to the CNS for the purpose of tumour treatment defined in D1 (cf. pt. 1). Consequently, present claims 1 and 7 lack novelty over the teaching of D1.

2.2 The feature of highly pure, endotoxin free alginate defined in the dependent claim 8 is implicit in the technical field of D1 (use of immunoisolation devices for implantation into the human body).

The same applies for the form of microbeads or beads (local delivery in the treatment of brain tumours at page 19), defined in claim 6, even though this term

is not explicitly disclosed in D1.

- 2.3 None of the documents cited in the International Search Report discloses encapsulated producer cells, wherein the expression of said molecule can be switched on and off by an external pharmacological agent as defined in claim 2.

The same applies for the use of in alginate encapsulated producer cells in the treatment of CNS tumours, where the alginate has a G content such as defined in the dependent claims 3-5.

Hence, claims 2-5 are considered as novel over the cited prior art.

3. Inventive step (Article 33(3) PCT)

- 3.1 The mere application of a common procedure for encapsulation of producer cells in this particular technical field, consisting of the drop-wise addition of a viable cell containing alginate solution to a calcium solution, constituting a one-step procedure, does not render claims 9 and 10 appended to claim 1 inventive. Moreover is it obvious to a skilled person that by applying this common procedure, an encapsulated cell is achieved pertaining higher alginate concentration at the outer boarder of the capsules, thus rendering claim 11 non-inventive.
- 3.2 D3 describes tetracycline-controlled gene expression. No reference is made to encapsulated cells in which gene expression is regulated. None of the other documents cited in the International Search Report refers to controlling gene expression by a pharmacological agent. The subject-matter of claim 2 is considered as non-obvious over the cited prior art.

The present application achieves sufficient immunoisolation by sole encapsulation of said producer cells in alginate with a specific G-content such as defined in claims 3-5. No hint is apparent from the cited documents which would lead the skilled person to sole the problem of immunoisolation of producer cells for the treatment of CNS tumours by solely encapsulating these cells in one alginate matrix (thus representing core and outer layer).

Thus, claims 2-5 fulfill the requirement of Article 33(3) PCT.

**INTERNATIONAL PRELIMINARY
EXAMINATION REPORT - SEPARATE SHEET**

International application No. PCT/NO99/00266

Re Item VIII

- A lack of clarity arises from the introduction of bracketed terms as well as by expressions such as "for example" (cf. claim 1 a), b) and c)) (Article 6 PCT).
- Claim 6 is insofar unclear, as the description defines producer cells encapsulated in the immuno-isolating matrices (p. 5, l. 1-5) preferably being microbeads such that it is understood that the encapsulated producer cells represent said beads/microbeads. However claim 6 envisages bead or microbeads enclosing ~~encapsulated~~ producer cells (Art. 6 PCT).
- The specific G contents of 60-80% and 80-100% of said alginate defined in claim 4 and 5 do not have any basis in the description (Art. 6 PCT)

CLAIMS

1. The use of a cell in the preparation of a medicament for treating a CNS tumour; wherein the medicament comprises an encapsulated producer cell capable of expressing a molecule which is an inhibitor of CNS tumour growth; wherein the encapsulation matrix is an immunoisolating alginate; and wherein said molecule is:

- a) a molecule that is capable of interacting with tumour/host communication pathways (e.g. a protein, a peptide or a polysaccharide);
- b) a molecule (e.g. a protein, a peptide or a polysaccharide) that is capable of affecting tumour neovascularization;
- c) a molecule (e.g. a protein, a peptide or a polysaccharide) that is capable of interfering with the relationship of the tumour cell with the extracellular matrix; or
- d) a monoclonal antibody capable of interacting directly with a receptor of the tumour, wherein said monoclonal antibody binds to or interacts with platelet derived growth factor receptors AA and BB, acidic and basic fibroblast growth factor receptors, transforming growth factor receptor alpha and beta, different classes of vascular endothelial growth factor receptors (VEGFR-1 and VEGFR-2), tyrosine kinase receptors with immunoglobulin-like and EGF-like domains such as, for instance, TIE-1 and TIE-2/tek, hepatocyte growth factor (scatter factor); CD-44; CDR/cyclin complexes; glycolipids on the cell surface; glycoproteins; and proteins derived from the expression of specific oncogenes.

2. The use according to claim 1, wherein said expression of said molecule is capable of being switched on and off by an external pharmacological agent.

3. The use according to claim 1 or claim 2, wherein the alginate has a G content of above 15%, preferably above 50%.

4. The use according to any preceding claim, wherein the alginate has a G content of 60%-80%.

5. The use according to any preceding claim, wherein the alginate has a G content of 80%-100%.

6. The use according to any preceding claim, wherein the encapsulated producer cell is present in a bead or a microbead.
7. The use according to any preceding claim, wherein said CNS tumour is a brain tumour.
8. The use according to any preceding claim, wherein the alginate is of a high-purity, endotoxin free quality.
9. The use according to any preceding claim, wherein the preparation of the medicament comprises encapsulating producer cells in a one-step procedure.
10. The use according to any preceding claim, wherein the preparation of the medicament comprises the drop-wise addition of an alginate solution containing viable cells to a calcium solution.
11. The use according to any preceding claim, wherein the encapsulated producer cell has an alginate concentration which rises to the outer rim of the capsule.

REC'D 29 DEC 2000

PCT



WIPO

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

15

Applicant's or agent's file reference P9803	FOR FURTHER ACTION See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)	
International application No. PCT/NO99/00266	International filing date (day/month/year) 25/08/1999	Priority date (day/month/year) 26/08/1998
International Patent Classification (IPC) or national classification and IPC A61K9/16		
Applicant NORSK HYDRO ASA et al		
<p>1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.</p> <p>2. This REPORT consists of a total of 8 sheets, including this cover sheet.</p> <p><input checked="" type="checkbox"/> This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).</p> <p>These annexes consist of a total of 2 sheets.</p>		
<p>3. This report contains indications relating to the following items:</p> <ul style="list-style-type: none">I <input checked="" type="checkbox"/> Basis of the reportII <input type="checkbox"/> PriorityIII <input type="checkbox"/> Non-establishment of opinion with regard to novelty, inventive step and industrial applicabilityIV <input checked="" type="checkbox"/> Lack of unity of inventionV <input checked="" type="checkbox"/> Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statementVI <input type="checkbox"/> Certain documents citedVII <input type="checkbox"/> Certain defects in the international applicationVIII <input checked="" type="checkbox"/> Certain observations on the international application		
Date of submission of the demand 18/03/2000	Date of completion of this report 27.12.2000	
Name and mailing address of the international preliminary examining authority:  European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Muller, I Telephone No. +49 89 2399 8716 	

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/NO99/00266

I. Basis of the report

1. This report has been drawn on the basis of *(substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments (Rules 70.16 and 70.17).)*:

Description, pages:

1-44 as originally filed

Claims, No.:

1-11 with telefax of 21/09/2000

Drawings, sheets:

1/7-7/7 as originally filed

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language: , which is:

- ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
- ☐ the language of publication of the international application (under Rule 48.3(b)).
- ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- ☐ contained in the international application in written form.
- ☐ filed together with the international application in computer readable form.
- ☐ furnished subsequently to this Authority in written form.
- ☐ furnished subsequently to this Authority in computer readable form.
- ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
- ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

- ☐ the description, pages:
- ☐ the claims, Nos.:

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/NO99/00266

☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

(Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

IV. Lack of unity of invention

1. In response to the invitation to restrict or pay additional fees the applicant has:

- ☐ restricted the claims.
☐ paid additional fees.
☐ paid additional fees under protest.
☐ neither restricted nor paid additional fees.

2. ☒ This Authority found that the requirement of unity of invention is not complied and chose, according to Rule 68.1, not to invite the applicant to restrict or pay additional fees.

3. This Authority considers that the requirement of unity of invention in accordance with Rules 13.1, 13.2 and 13.3 is

- ☐ complied with.
☒ not complied with for the following reasons:
see separate sheet

4. Consequently, the following parts of the international application were the subject of international preliminary examination in establishing this report:

- ☒ all parts.
☐ the parts relating to claims Nos. .

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	2-5,9-11
	No:	Claims	1,6-8
Inventive step (IS)	Yes:	Claims	2-5
	No:	Claims	1,6-11

**INTERNATIONAL PRELIMINARY
EXAMINATION REPORT**

International application No. PCT/NO99/00266

Industrial applicability (IA) Yes: Claims 1-11
 No: Claims

2. Citations and explanations
see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:
see separate sheet

**INTERNATIONAL PRELIMINARY
EXAMINATION REPORT - SEPARATE SHEET**

International application No. PCT/NO99/00266

Re Item I

The disclaimer introduced in the sole independent claim 1 filed with letter of 21.11.00 received on 22.12.00 is not acceptable as D1, relating to the same technical field, cannot be considered as accidentally novelty destroying (Art. 19(2) PCT). Moreover, the fact that apoptosis of tumour cells is not explicitly referred to within the application documents does not exclude such apoptosis from the subject-matter of the application.

As a result this international preliminary examination report is drawn on the basis of the amendments filed by the applicant via telefax on 21.09.00.

Re Item IV

The subject-matter of independent claim 1 is already known (cf. item V). The requisite unity of invention (Rule 13.1 PCT) therefore no longer exists inasmuch as a technical relationship involving one or more of the same or corresponding special technical features in the sense of Rule 13.2 PCT does not exist between the subject-matter of the alternatives b) and d) defined in present claim 1 (Rule 13.3 PCT).

Re Item V

1. Reference is made to the following documents:

D1: WO-A1-9738707

D2: WO-A1-9628029

D3: Dong X. Yin, Li Zhu, R.T. Schimke, Tetracycline-Controlled Gene Expression System Achieves High-Level and Quantitative Control of Gene Expression. Analytical Biochemistry 1996, Vol. 235, No 0112, Pages 195-201.

D1 discloses devices for delivery of a cellular source of an apoptosis- inducing molecule (cf. claim 1 and FasL at. page 2, line 23 - page 3, line 11 and page 5, line 18 - page 6, line 12). The preferred devices used for the encapsulation of the cells have to fulfill the requirement of immunoisolation (p. 14, l. 29-30; p. 15, l. 1-6 and p. 16, l. 10-12) and may consist of alginate (p. 15, l. 9-14 and 29-30). At page 19, l. 10-11 reference is made to the treatment of brain tumours by said encapsulated source of FasL.

D2 discloses the immunoisolation of cells expressing therapeutic substances in composite microreactors (microcapsules) (p. 2, l. 3-5). One of the preferred

encapsulation matrices is alginate (p. 3, l. 13-14), the microreactors are in form of microbeads or microspheres (p. 65, l. 10-12). In this document moreover implantation of the beads into the brain (p. 74, l. 9-10) is envisaged, beads providing alpha-interferon are described at p. 74, l. 13-15 and treatment of several cancer forms (solid tumours, hair cell leukemia, Kaposi's sarcoma etc..) by microcapsules containing cells expressing alpha-interferon is described (p. 84, l. 21-33). No reference is made to using in alginate immunoisolated producer cells for treating specifically CNS tumours. In order to achieve effective immunoisolation properties, an outer layer is required to prevent cracking and subsequent host cell penetration through the encapsulation matrix (ex. 1, p. 75, l. 12-13).

2. Novelty (Article 33(2) PCT)

Claim 1 defines the use of a cell in general. It is not clear from the formulation of the claim that this cell is identical to the producer cell comprised in the defined medicament. Notwithstanding in the following, said cell is understood as the 'producer cell' defined by the applicant.

2.1 The wording "inhibition of tumour growth" in its broad meaning includes both slight slow down of tumour growth and prevention of tumour growth. The latter may consist for example in induced apoptosis of the tumour cells.

Moreover can the effect of the FasL protein on CNS tumour be classified under interaction with tumour/host communication pathways, said cell apoptosis influencing in some way these communication pathways. The same is applicable for the interference with the relationship of the tumour cell with the extracellular matrix. Thus, present claim 1 does not define any technical features providing distinction from the delivery device of producer cells expressing FasL protein to the CNS for the purpose of tumour treatment defined in D1 (cf. pt. 1).

Consequently, present claims 1 and 7 lack novelty over the teaching of D1.

2.2 The feature of highly pure, endotoxin free alginate defined in the dependent claim 8 is implicit in the technical field of D1 (use of immunoisolation devices for implantation into the human body).

The same applies for the form of microbeads or beads (local delivery in the treatment of brain tumours at page 19), defined in claim 6, even though this term

is not explicitly disclosed in D1.

- 2.3 None of the documents cited in the International Search Report discloses encapsulated producer cells, wherein the expression of said molecule can be switched on and off by an external pharmacological agent as defined in claim 2.

The same applies for the use of in alginate encapsulated producer cells in the treatment of CNS tumours, where the alginate has a G content such as defined in the dependent claims 3-5.

Hence, claims 2-5 are considered as novel over the cited prior art.

3. Inventive step (Article 33(3) PCT)

- 3.1 The mere application of a common procedure for encapsulation of producer cells in this particular technical field, consisting of the drop-wise addition of a viable cell containing alginate solution to a calcium solution, constituting a one-step procedure, does not render claims 9 and 10 appended to claim 1 inventive. Moreover is it obvious to a skilled person that by applying this common procedure, an encapsulated cell is achieved pertaining higher alginate concentration at the outer boarder of the capsules, thus rendering claim 11 non-inventive.
- 3.2 D3 describes tetracycline-controlled gene expression. No reference is made to encapsulated cells in which gene expression is regulated. None of the other documents cited in the International Search Report refers to controlling gene expression by a pharmacological agent. The subject-matter of claim 2 is considered as non-obvious over the cited prior art.

The present application achieves sufficient immunoisolation by sole encapsulation of said producer cells in alginate with a specific G-content such as defined in claims 3-5. No hint is apparent from the cited documents which would lead the skilled person to sole the problem of immunoisolation of producer cells for the treatment of CNS tumours by solely encapsulating these cells in one alginate matrix (thus representing core and outer layer).

Thus, claims 2-5 fulfill the requirement of Article 33(3) PCT.

R Item VIII

- A lack of clarity arises from the introduction of bracketed terms as well as by expressions such as "for example" (cf. claim 1 a), b) and c)) (Article 6 PCT).
- Claim 6 is insofar unclear, as the description defines producer cells encapsulated in the immuno-isolating matrices (p. 5, l. 1-5) preferably being microbeads such that it is understood that the encapsulated producer cells represent said beads/microbeads. However claim 6 envisages bead or microbeads enclosing encapsulated producer cells (Art. 6 PCT).
- The specific G contents of 60-80% and 80-100% of said alginate defined in claim 4 and 5 do not have any basis in the description (Art. 6 PCT)

CLAIMS

1. An encapsulated producer cell capable of expressing a molecule which is an inhibitor of CNS tumour growth, wherein the encapsulation matrix is an immuno-isolating alginate.

2. An encapsulated producer cell according to Claim 1, wherein said molecule is a protein or peptide capable of interacting with tumour/host communication pathways.

3. An encapsulated producer cell according to Claim 2, wherein said protein or peptide is capable of affecting tumour neovascularization.

4. An encapsulated producer cell according to Claim 2, wherein said protein or peptide is capable of interfering with the relationship of the tumour cell with the extracellular matrix.

5. An encapsulated producer cell according to Claim 1, wherein said molecule is a protein or peptide capable of interacting directly with the tumour cell.

6. An encapsulated producer cell according to Claim 5, wherein said producer cell is a hybridoma cell capable of expressing a monoclonal antibody capable of interacting directly with a receptor of the tumour.

REPLACED BY
ART 84/2001

7. An encapsulated producer cell according to any preceding claim, wherein said expression of said molecule is capable of being switched on and off by an external pharmacological agent.

8. An encapsulated producer cell according to claim 1, wherein the alginate has a G content of above 15%, more preferred above 50%.

9. An encapsulated producer cell according to Claim 8, wherein the alginate has a G content of 60%-80%.

10. An encapsulated producer cell according to Claim 1, wherein the alginate has a G content of 80%-100%.

11. An encapsulated producer cell according to any one of Claim 1 in the form of beads or microbeads.

12. An encapsulated producer cell according to any preceding claim, wherein said tumour is a brain tumour.

13. A method for the treatment of CNS tumour, which comprises implanting at the site of the tumour an immuno-isolating alginate encapsulated producer cell which is capable of expressing a molecule which is an inhibitor of the growth of said tumour.

14. A method according to Claim 13, wherein said encapsulated producer cell is as defined in any one of Claims 1-12.

REPLACED BY
ART 34 AMDT

15. A method according to Claim 13 or Claim 14, wherein said producer cells are implanted following surgical removal of said tumour.

16. A method for the preparation a pharmacological product for the treatment of a CNS tumour, which comprises encapsulating within an immuno-isolating matrix a producer cell capable of expressing a molecule which is an inhibitor of the growth of said tumour.

17. A use of an immunoisolating alginate for preparing encapsulated producer cells intended for transplantation in the brain after surgical removal of a brain tumour, which producer cell capable of expressing a molecule which is an inhibitor of the growth of said tumour.

18. A use according to claim 17, wherein the alginate is of a high-purity, endotox in free quality.

19. A use according to claim 17, wherein the alginate has a G content of at least 15%, more preferred above 50%.

REPLACED BY
ART 34 AMDT

PCTWORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁷ : A61K 9/16, A61P 35/00 // C12N 11/04	A1	(11) International Publication Number: WO 00/12066 (43) International Publication Date: 9 March 2000 (09.03.00)
(21) International Application Number: PCT/NO99/00266 (22) International Filing Date: 25 August 1999 (25.08.99) (30) Priority Data: 19983911 26 August 1998 (26.08.98) NO (71) Applicant (for all designated States except US): NORSK HYDRO ASA [NO/NO]; N-0240 Oslo (NO). (72) Inventor; and (75) Inventor/Applicant (for US only): BJERKVIG, Rolf [NO/NO]; Skarehaugene 40B, N-5070 Mathopen (NO). (74) Agent: SANDBU, Elisabeth, Dahl; Norsk Hydro ASA, N-0240 Oslo (NO).		(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
(54) Title: ALGINATE CAPSULES FOR USE IN THE TREATMENT OF BRAIN TUMOUR (57) Abstract Encapsulated producer cells which are capable of expressing a molecule which is an inhibitor of CNS tumour growth provide a novel approach to the treatment of tumours, such as brain tumours which are localized within the central nervous system.		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece			TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon			PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

Alginate capsules for use in the treatment of brain tumour

5

This invention is in the field of the treatment of tumours which are localized within the central nervous system (CNS) and of primary and secondary (metastatic) cerebral-spinal malignancies, and it provides new
10 compositions and delivery systems useful in such therapy.

Primary brain tumours (gliomas) have several unique biological features compared to other metastatic tumours. They are confined within the central nervous
15 system and metastatic spread to other organs is virtually non-existent. Even though these tumours show a high degree of invasion into the brain they have a tendency to recur after treatment in positions where they originally were found. The tumours are highly heterogeneous and
20 consist of numerous cell types with different phenotypic properties.

At present the treatment of choice is surgery followed by radiotherapy and chemotherapy. Patients with
25 the most malignant forms of brain tumours (glioblastomas) have a severe prognosis with a survival of approximately 10 months after diagnosis. There is therefore an urgent need for new treatment strategies for this particular group of tumours. Since the tumours have a tendency to
30 recur at its primary site, new local treatment strategies are needed. Furthermore since these tumours consist of

numerous tumour cells with different phenotypic properties, the treatment of choice should be capable of targeting different tumour cell types.

- 5 Other tumours which are localized within the central nervous system and which are often difficult to treat successfully include tumours derived from astroglial and oligodendroglial cells, for instance:

10

Astrocytomas

- Low grade astrocytomas (astrocytomas grade 1 and 2)
- Anaplastic astrocytoma (astrocytoma grade 3)
- 15 - Glioblastoma multiforme (astrocytoma grade 4)
 - including secondary glioblastoma, i.e. tumours that have differentiated from astrocytomas with lower grade
- 20 - primary glioblastoma, i.e. tumours that occur as primary glioblastomas *de novo*
- giant cell glioblastoma
- gliosarcomas
- gliomatosis cerebri

25

Oligodendrogliomas

- including oligodendroglioma (WHO grade II)
- anaplastic oligodendroglioma (WHO grade III)

Mixed Gliomas

- Oligoastrocytoma (WHO grade II)
- Anaplastic oligoastrocytoma (WHO grade III)

5 Ependymal tumours

- Ependymoma (WHO grade II)
- Anaplastic ependymoma (WHO grade III)
- Subependymoma (WHO grade I)

10 Embryonal tumours

- Central neuroblastoma
- Ependymoblastoma
- Medulloblastomas
- Supratentorial PNETs

15

Neuroblastomas

- Olfactory neuroblastoma
- Neuroblastic tumours of the adrenal gland and sympathetic nervous system

20

For most of these tumours, the first treatment of choice is surgery followed with radiotherapy and/or with chemotherapy. However, complete tumour removal is often difficult by surgical procedures, whilst follow-up radiotherapy and chemotherapy are also sometimes not completely successful due to radioresistance and/or difficulties in delivering therapeutic doses of cytotoxic drugs.

During recent years much attention has been focused on gene therapy, where reversion of the malignant phenotype by downregulation of oncogene expression or insertion of normal tumour-suppressor genes have been

tried. Immune stimulatory factors such as cytokines that are designed to enhance the recognition and rejection of tumours by the immune system have also been introduced. Furthermore, cells have been modified to allow direct
5 delivery of gene products to tumour cells, increasing their susceptibility to pharmacological agents. Papers which describe these developments include (i) Curr Opin Oncol, 7, (1995), pages 94-100; (ii) Curr Opin Biotechnol, 5, (1994), pages 611-616; (iii) Cancer Res,
10 53, (1993), pages 2330-7; (iv) Hum Gene Ther, 4, (1993), pages 451-60; (v) Hum Gene Ther, 5, (1994), pages 153-164; and (vi) Trends Pharmacol. Sci, 14, (1993), pages 202-208.

15 Despite this extensive research during recent years, there are major obstacles which impede the transition between experimental research and clinical treatment of malignant brain tumours. One problem is to prevent immuno-rejection of genetically modified cells
20 after intracranial implantation. This may be overcome by encapsulating the producer cells.

 However, this results in other problems, to find materials especially adapted for use in the brain.
25 Although, the brain is immunologically different from other areas of the body, for instance in its lack of B lymphocytes, it is especially sensitive to the influence of biologically active compounds such as for instance endotoxins.

30

 We have now found, in accordance with the present invention, that immuno-isolating alginate

matrices are especially suitable for the encapsulation of producer cells intended for implantation intercranially, in the treatment of CNS tumours. It is especially preferred that the immuno-isolating alginate matrices should be microbeads.

Thus, in its broad aspect, the present invention provides an encapsulated producer cell capable of expressing a molecule which is an inhibitor of CNS tumour growth, which producer cell is encapsulated in an immuno-isolating alginate matrix. It is preferred that this molecule should be a peptide, a protein or a polysaccharide and most preferred the molecule is a monoclonal antibody.

15

The present invention also provides a method for the treatment of CNS tumours, which comprises implanting at the site of the tumour an encapsulated producer cell which is capable of expressing a molecule which is an inhibitor of the growth of said tumour.

Further, the present invention provides a method for the preparation a pharmacological product for the treatment of a CNS tumour, which comprises encapsulating within an immuno-isolating alginate matrix a producer cell capable of expressing a molecule which is an inhibitor of the growth of said tumour.

The present invention also provides for the use of immuno-isolating alginate matrices for encapsulation of producer cells intended for implantation intercranially, in the treatment of CNS tumours.

In one embodiment of the invention the producer cells contemplated for use herein include genetically engineered cells that produce molecules e.g. proteins, peptides and polysaccharides, that will either directly
5 interact with tumour cells or indirectly with tumour or host cell communication pathways. Other useful producer cells contemplated herein are specialized cells which produce monoclonal antibodies as for instance hybridoma cells, or even naturally occurring cells which are
10 capable of expressing tumour inhibiting molecules.

It is well known that tumour growth is dependent on specific cellular interactions with the host, mediated via specific growth factors that regulate
15 tumour cell growth in rather complex ways. The tumours depend in this respect on nutrients mediated via newly formed blood vessels supplied by the host. Several tumour/host cellular interaction pathways have during the last years been identified and described in the
20 literature.

Accordingly, one class of producer cell useful herein are those which can express proteins or peptides that will interact with tumour/host communication
25 pathways. For instance, useful producer cells include those which produce proteins and peptides which affect tumour neovascularization as for instance thrombospondin, endostatin, angiostatin and prolactin, proteins which interfere with the tumour cells' relationship to the
30 extracellular matrix, for instance protease inhibitors such as tissue inhibitors of metalloproteinases, and proteins and peptides which affect the immune system,

including all the various classes of interleukins.

Another preferred class of producer cell is constituted by those which express proteins or peptides which interact directly with the tumour cells themselves. For instance, useful producer cells of this category include: hybridoma cell lines that produce monoclonal antibodies which interact directly with a receptor of the tumour, for example cell growth factor receptors which affect the tumour cells such as epidermal growth factor receptor (EGFr), platelet derived growth factor receptors AA and BB, acidic and basic fibroblast growth factor receptors, transforming growth factor receptor alpha and beta, the different classes of vascular endothelial growth factor receptors (VEGFR-1 and VEGFR-2), tyrosine kinase receptors with immunoglobulin and EGF-like domains as, for instance, TIE-1 and TIE-2/tek, heptaocyte growth factor (scatter factor); or monoclonal antibodies directed against various classes of integrin receptors; monoclonal antibodies directed against CD-44; monoclonal antibodies directed against CDK/cyclin complexes; monoclonal antibodies directed against FAS; monoclonal antibodies directed against glycolipids on the cell surface; monoclonal antibodies directed against glycoproteins; and monoclonal antibodies directed against proteins derived from the expression of specific oncogenes.

Of particular interest in some circumstances are producer cells whose production of tumour growth-inhibiting substances can be switched on and off by pharmacological means, for instance producer cells with

pharmacologically-inducible gene expression as, for example, tetracycline-activated gene expression.

Any cell line which is transfectable may be used in accordance with this invention. The cell lines should be permanent, i.e. able to undergo unlimited cell division, and preferably are non-human and non-tumorigenic.

10 Examples of such cell lines which are freely commercially available from the American Type Culture Collection, 10350 Linden Lake Plaza, Manassas, Virginia 20109, USA, are:

<u>Cell Line</u>	<u>ATCC number</u>	<u>Description</u>
H528	HB 8509	mouse B cell myeloma
293	CRL 1573	human transformed primary embryonal kidney
NIH/3T3	CRL 1658	NIH swiss mouse, embryo
COS-7	CRL 1651	African green monkey, kidney, SV40 transform
BHK-21	CCL 10	Hamster kidney, normal
CV-1	CCL 70	African green monkey, kidney, normal
CHP-234	CRL-2272	Neuroblastoma, brain, human
Rat2	CRL-1764	Embryo, thymidine kinase mutant, rat
Namalwa	CL-1432	Burkitt's lymphoma, human

In accordance with the present invention, the producer cells are encapsulated in immuno-isolating
5 alginate matrices which are capable of providing a stable, *in situ* delivery system of expressed protein or other molecule which can interfere with tumour growth and progression without immuno-rejection of the producer cells.

10

The encapsulation of cells within alginate beads is a well known technique for immobilising cells and other substances, and has previously been used in the treatment of diabetes mellitus, in the production of
15 monoclonal antibodies, and in other medical areas, as has been described in the literature.

From PCT/WO97/44065 this present drug delivery technique has been proposed for in vivo gene therapy
20 using encapsulated cells releasing gene transfer vectors at the site of a brain tumour. The capsules used for encapsulating the cells comprise two parts: a) a core comprising living packaging cells and b) an external jacket surrounding said core.

25

The present invention provides for a much simpler encapsulation process and product, wherein the producer cells are encapsulated directly in a one-step procedure using an immuno-isolating alginate quality.

30

Alginate is a polysaccharide which is mainly

found in brown seaweed. It consists of two types of monosaccharides; L-guluronic acid (G) and D-mannuronic acid (M). These polysaccharide units appear in blocks of alternating sequences of G and M (MG-blocks) and blocks
5 mainly consisting of either G or M units (G-blocks/M-blocks).

The gelforming property is achieved through a cross-link of G-blocks with multivalent cations,
10 especially Ca^{2+} .

In order for an alginate not to be immunologically activating the G content must be above 15%. However it is more preferred, according to the present
15 invention to use a high G alginate, i.e. with a G content of 50% or more in order to ascertain that the alginate is immuno-isolating. As is well-known in the art the G/M-block ratios and the distribution of the different blocks are critical factors for the different properties of the
20 resulting gel formed through cross-linking with a polyvalent cation.

Another aspect which is critical is the purity of the alginate to be used. Thus, one advantage of the
25 alginate matrices usable according to the present invention is that they may be produced in a high-purity quality having a well defined constitution and a very low content of impurities such as endotoxins.

30 A second advantage of the alginate matrices usable according to the present invention is that alginate microbeads prepared by the drop-wise addition of

an alginate solution containing viable cells to a calcium solution, have a rising alginate concentration from the center of the microbead to the outer rim. Thereby an optimal space is created at the center of the microbeads for the cells to live, proliferate and produce whereby sufficient nutrients and oxygen is available to the cells. The outer rim with its higher alginate concentration gives rise to a barrier, so that the producing cells inside the microbeads do not escape from the interior, nor do immunological cells enter into the beads.

Generally, the use of alginate as an immobilisation matrix for cells involves mixing a suspension of the cells with a Na⁺ alginate solution, whereafter the mixture is dripped into a solution containing multivalent cations (usually Ca²⁺). The droplets form gel spheres instantaneously entrapping the cells in a three-dimensional lattice of ionically crosslinked alginate. This immobilisation procedure can be carried out under very mild conditions and is therefore compatible with most living cells. For a detailed description both of the theory and practice of the technique, the reader is directed to the paper "Alginate as Immobilization Matrix for Cells" by Smidsrød and Skjak-Braek in Trends in Biotechnology, March 1990, Vol. 8, No. 3, pages 71-78.

A currently preferred method for forming producer cell-encapsulated calcium alginate beads in accordance with this invention is as follows. Sodium alginate is dissolved at a concentration of from 1-2% in

water or isotonic saline. The alginate solution is membrane sterilized, and the producer cells are then added and isotonicity adjusted. Calcium alginate beads are formed by dripping the sodium alginate-producer cell solution into a bath of calcium chloride (0.05-0.25 M), either manually but preferably using an electrostatic bead generator which establishes an electrostatic potential of 5 to 7 kV between the alginate feed needle and the gelling bath. By adjusting the needle diameter (e.g. from 0.1 mm to 0.4 mm), the flow rate (e.g. from 5 ml/hr to 30 ml/hr) and the voltage applied, beads of comparatively uniform diameter of from 100-400 μ m can be generated. The homogeneity of the beads is controlled by adjusting the salt concentration in the gelling bath, from 0 to 200 mM NaCl, with the higher salt concentration giving greater homogeneity. The beads are allowed to harden in the gelling bath.

It is contemplated that the encapsulated producer cells of this invention will be placed into the tumour cavity following conventional bulk tumour removal by surgery. Shortly after surgery the tumour burden is minimal and many patients have a symptom-free period before recurrence occurs. Since surgery is a traumatic event, the remaining tumour cells will try to establish new biochemical interaction pathways with the host. This involves the formation of new blood vessels and new supplies of peptide growth factors to the remaining tumour cells. It is at this time, when the tumour burden is at a minimum, that the treatment made possible by the present invention is most likely to be effective.

It is, indeed, a particular advantage of the

present invention in accordance with one embodiment that it readily permits the simultaneous implantation of several different types of producer cells to target different phenotypic characteristics and

5 microenvironmental factors influencing the progressive growth of brain or other tumours. For this purpose, a producer cell bank containing encapsulated producer cells stored frozen at the temperature of liquid nitrogen could be established. Producer cells could then be withdrawn

10 from the bank to meet the genotypic expression of the host tumour being treated.

In order to establish what producer cells are required for treatment of a tumour the following

15 procedure could be used, by way of example. Tumour characterization involving determination of receptor status and phenotype is first performed on biopsy material. Appropriately chosen producer cells which produce substances, for example monoclonal antibodies,

20 directed against the receptor status of the host tumour is then implanted stereotactically up to 60 days following surgical removal of the primary tumour.

Alternatively, producer cells producing anti-

25 angiogenic substances can be implanted directly following surgical removal of the primary tumour.

The dosage of producer cells to be implanted will, of course, depend on precise circumstances of each

30 patient, but typically the total number of implanted cells would be in the range from 10^6 to 10^{12} per patient. The number of producer cells within each alginate or

other encapsulating matrix will, of course, depend on the dimensions of the bead or other encapsulating form. The encapsulated producer cells will generally be surgically placed at the wound site following removal of
5 the primary tumour.

As the experiments to be described in detail below have shown, encapsulated producer cells can survive, proliferate and maintain their specific
10 expression periods *in vitro* and *in vivo*. This discovery opens up the possibility of a new kind of therapeutic treatment for patients with brain tumour conditions whereby different producer cells may be encapsulated which are chosen so as to target selected characteristics
15 of brain tumour growth and development. In the experiments described herein, we have shown that specific MAbs released from alginate beads can inhibit tumour cell migration as demonstrated by an interference with epidermal growth factor receptor. We have also shown that
20 specific products released from encapsulated producer cells within the brain penetrate into the brain parenchyma and can be distributed along CSF pathways.

The following experiments will assist in
25 understanding the invention and its advantages. Hereafter reference will be made to the accompanying drawings, in which:

Figures 1A-1C

Light microscopic images of NIH 3T3 cells encapsulated in alginate. All bars represent 250 μm .

5

Fig. 1A: The day of encapsulation.

Fig. 1B: Encapsulated cells after 3 weeks in culture.

10 Fig. 1C: Encapsulated cells after 9 weeks in culture.

Figs. 1D-1F: Scanning confocal laser micrographs of NIH 3T3 cells encapsulated in alginate. Viable cells emit green fluorescence (here shown as lighter areas), while dead cells emit red fluorescence (not here visible). All bars represent 250 μm .

15

Fig. 1D: At the day of encapsulation.

20

Fig. 1E: Encapsulated cells after 3 weeks in culture.

Fig. 1F: Encapsulated cells after 9 weeks in culture.

25 Fig. 1G: β -Galactosidase activity of BT4CnVlacZ cells encapsulated in alginate, after 9 weeks in culture. Bar represents 500 μm .

Figures 2A-2D

30

Flow cytometric histograms of NIH 3T3 cells encapsulated in alginate beads. The horizontal axis

expresses the number of channels on the flow cytometer (relative DNA fluorescence), while the vertical axis expresses the relative number of cell nuclei in each channel.

5

Fig. 2A: Control, monolayer culture.

Fig. 2B: Cells encapsulated for 1 week.

10

Fig. 2C: Cells encapsulated for 3 weeks.

Fig. 2D Cells encapsulated for 9 weeks.

Figure 3

15

Antibody release from H528 hybridoma cells encapsulated in alginate (mean value \pm standard error). The horizontal axis represents the number of days in culture, while the vertical axis shows the antibody
20 release into the growth medium. The curve was estimated by a 3rd order regression analysis.

Figure 4

25

Migration of cells from GaMg spheroids after 4 days, untreated (control), stimulated with 10 ng/ml EGF (EGF), or stimulated with 10 ng/ml EGF in the presence of encapsulated hybridoma cells (EGF/H528).

Figures 5A-5H

Encapsulated H528 hybridoma cells implanted into the rat brain.

5

Fig. 5A: Axial section of the rat brain.
H&E-staining, bar represents 5 mm.

10

Fig. 5B: Same section as Fig. 5A, showing
encapsulated H528 cells inside the
implantation site. H&E-staining, bar
represents 500 μm .

Figs. 5C-5H: Confocal laser scanning micrographs of the
release and dissemination of monoclonal antibodies within
the brain. Figs. 5C, E and F were taken with identical
gain settings. Figs. 5G and 5H were also taken with
identical gain settings.

20

Fig. 5C: A section of the brain parenchyma, with the
encapsulated H528 cells at the far left
side. Bar represents 150 μm . An intense
fluorescence in the brain parenchyma is seen
at the left side, followed by a gradual
decrease in intensity at least 1000 μm into
the brain.

25

The gradual change in fluorescence intensity
along the horizontal line is further shown in Fig. 5D
where the vertical axis represents the relative
fluorescence intensity (0-255). An intense fluorescence
is seen from the left side, with a gradual decrease into
the brain parenchyma.

30

- Fig. 5E: The MAbs were found in the subarachnoidal space and in the underlying brain. Bar represents 75 μm .
- 5
- Fig. 5F: The weak fluorescence presented in the controls was probably caused by unspecific binding. Bar represents 75 μm .
- 10 Fig. 5G: MAbs were further spread within the perivascular space. Bar represents 50 μm .
- Fig. 5H: In comparison, the controls showed a weak binding of immunoglobulins in the
- 15 perivascular space. Bar represents 50 μm .

Fig. 6: Radioimmunoassays that shows the successful establishment of endostatin producer cells.

5 The figure shows radioimmunoassays of endostatin release from conditioned medium, in cellular fractions and medium from untransfected cells in the second, third and the fourth column, respectively.

Fig. 7: Effects of endostatin alginate therapy on tumour growth. Panel A shows example of a control animal where mock transfected cells encapsulated in alginate beads were implanted. The darker area of the brain shows the tumour area.

15 Panel B shows example of an animal treated with encapsulated endostatin producing cells. The darker area shows the tumour, and a large necrotic area is visualised in the middle of the tumour.

20

EXPERIMENTS

MATERIALS AND METHODS

5 1. Cell lines

In our experiments, four different cell lines were used:

10	<u>Cell Line</u>	<u>Deposit Details</u>
	1. NIH 3T3	ATCC CRL/1658
	2. BT4CnVlacZ	Not deposited
	3. H528ATCC HB 8509	
	4. GaMgNot deposited	

15

The mouse fibroblast NIH 3T3 cells represents a potential producer cell line in that it is capable of being genetically engineered to express substances which show effects against tumour growth, progression and development. The NIH 3T3 cells were encapsulated in alginate, as described below and used to study *in vitro* morphology, viability and cell kinetics. For studies of the viability of encapsulated cells *in vivo*, alginate beads containing NIH 3T3 cells were also implanted into the rat brain.

The BT4CnVlacZ cell line was originally developed from an ethylnitrosourea induced rat glioma and stably transfected with the bacterial *lacZ* gene, cloned into a plasmid containing a Moloney murine leukaemia virus long terminal repeat cassette with a neomycin resistance gene expressed from an internal Rous sarcoma

virus promoter. See J. Natl Cancer Inst, 55 (1975), pages 1177-87 and Int. J. Cancer, 71 (1997), pages 874-80. The cells were encapsulated in alginate, and the *in vitro* synthesis of the bacterial β -galactosidase was studied.

5

The H528 hybridoma cell line was obtained from American Type Culture Collection (ATCC Rockville, MA). The cell line was generated by fusing NS-1-Ag4-1 myeloma cells with spleen cells from BALB/c mice, and it produces
10 a mouse monoclonal antibody (MAb) (IgG2a) that binds to and blocks the EGF-binding domain of the human epidermal growth factor receptor (EGFR). The *in vitro* and *in vivo* MAb release from the alginate encapsulated cells was studied using this cell line.

15

The human glioma cell line GaMg has been described in Anticancer Res, 8 (1988) pages 874-80, and has previously been shown to express the EGFR (Acta Neuropathol Berl, 84 (1992), pages 190-197. The specific
20 inhibition of GaMg cell migration was studied in a co-culture system between GaMg multicellular spheroids and encapsulated H528 cells.

2. Cell culture

25

The NIH 3T3 and the BT4CnVlacZ cell lines were grown in 80 cm² culture flasks (Nunc, Roskilde, Denmark) with complete growth medium consisting of Dulbecco's modified Eagles medium (DMEM) supplemented with 10% heat
30 inactivated newborn calf serum, four times the prescribed concentration of non-essential amino acids, 2% L-Glutamine, penicillin (100 IU/ml) and streptomycin

(100 μ g/ml) (all biochemicals from BioWhittaker, Verviers, Belgium). The H528 hybridoma and the GaMg cell lines were grown in 80 cm² culture flasks (Nunc) in RPMI 1640 growth medium supplemented with 10% horse serum (BioWhittaker). GaMg monolayers were trypsinized at confluence with 3 ml of 0.025% trypsin (BioWhittaker), and spheroids were initiated by seeding 5×10^6 cells in 20 ml of complete RPMI medium into 80 cm² culture flasks (Nunc) base-coated with 0.5% agar noble (Difco, Detroit, MI) (30) in complete RPMI medium. All cell lines were kept in a standard tissue culture incubator at 37°C, with 100% relative humidity, 95% air and 5% CO₂.

3. Structure and properties of alginate

In these experiments sodium alginate from the brown seaweed *Laminaria hyperborea* (LF 10/60) (Protanal, Drammen, Norway) was used for microencapsulation of the producer cells. This consists of two monosaccharides; α -L-guluronic acid (G) and β -D-mannuronic acid (M). The G- and M-units are joined together in three different types of blocks, GG, MM and MG, and the proportions and distributions of these blocks determine the chemical and physical properties of the alginate molecules. Some divalent cations like CA²⁺ bind strongly between separate G-blocks, which initiate the formation of an extended alginate network where the G-blocks form stiff junctions. The alginate which we used has a high content, above 60%, of G-blocks, resulting in high mechanical stability and porosity, rendering it suitable for encapsulating cells for production of secondary metabolites (see Trends in

Biotechnology, 8 (1990), pages 71-78). Scanning electron microscopy has showed pore sizes in the alginate beads to range between 5 and 200 nm (33,34). Mechanical strength, volume stability and porosity of the beads correlate to the content of guluronic acid.

4. Encapsulation of cells

The method of encapsulation used has been described in detail in "Alginate as Immobilization Matrix for Cells" by Smidsrød and Skjak-Braek in Trends in Biotechnology, March 1990, Vol. 8, No. 3, pages 71-78.

Briefly, droplets of cells dispersed in 1.5% sodium alginate were released into a 0.1M Ca^{2+} -solution. After polymerization, the alginate beads were washed three times in Dulbecco's PBS (DPBS; Sigma, St. Louis, MO), and once in growth medium. The encapsulated cells were cultured in 175 cm² culture bottles (Nunc), containing 50 ml growth medium. The growth medium was changed every third day, and the bottles were replaced once a week. All alginate encapsulated cells were kept in a standard tissue culture incubator at 37°C, with 100% humidity, 95% air and 5% CO₂. For all the experiments with the NIH 3T3 and the BT4CnVlacZ cell lines, a cell density of $6 \cdot 10^6$ cells/ml alginate and bead sizes between 0.8 and 1.2 mm were used. For the *in vitro* experiments with the H528 cell line a cell density of $3 \cdot 10^5$ cells/ml alginate and bead diameters between 2.3 and 2.5 mm were used. For the *in vivo* experiments with the H528 cell line, a cell density of $3 \cdot 10^5$ cells/ml alginate and bead diameters between 0.8 and 1.2 mm were used.

IN VITRO EXPERIMENTS**1. Morphology and viability of alginate encapsulated cells**

5 The morphology of NIH 3T3 cells encapsulated in
alginate was investigated at the day of encapsulation,
and after 3 and 9 weeks, in 6 beads transferred to a
6-well dish (Nunc) with an overlay of 1.0 ml DPBS.
The beads were examined with a Nikon Diaphot light
10 microscope, and photographed with a Nikon F-301 camera.
The morphology experiments were performed in duplicate.

 The viability of the cells within the alginate
beads was investigated at the day of encapsulation, and
15 after 3 and 9 weeks, by a two-colour fluorescence
viability assay (Live/Dead™ Viability/Cytotoxicity Assay,

Molecular Probes, Eugene, OR). A labelling solution was
prepared with 2 μ M calcein-AM and 4 μ M ethidium homodimer
20 in complete growth medium. The alginate beads were
individually placed in 16-mm multiwell dishes (Nunc) with
an overlay of 0.5 ml labelling solution of 30 minutes at
room temperature. Thereafter they were transferred into
DPBS and examined immediately. The fluorescence was
25 measured in optical sections through the alginate using a
confocal laser scanning microscope with an argon-krypton
laser (Biorad MRC-1000, Hemel Hempstead, England), using
Texas Red and FITC filter optics. Fluorescence was
recorded in a plane 120 μ m inside the alginate beads. The
30 viability experiments were performed in triplicate.

The production of β -galactosidase in BT4CnVlacZ cells encapsulated in alginate for 1, 3 and 9 weeks was studied. The beads were washed for 1 minute in DPBS (pH = 8.4), and fixed for 10 minutes in 0.2% glutaraldehyde and 5 2% formaldehyde in DPBS. Thereafter they were washed 3 x 5 minutes in DPBS and stained for β -galactosidase activity with 5-bromo-4-chloro-3-indolyl β -D-galactopyranoside (x-gal; Sigma). The substrate solution consisted of 1 mg/ml x-gal dissolved in 100 μ l 10 dimethylformamide, and mixed with 5 mM potassiumferri-cyanite, 5 mM potassiumferrocyanate and 2 mM $MgCl_2$, dissolved in DPBS (all biochemicals from E. Merck, Darmstadt, Germany). They were incubated at 4°C for minimum 24 hours, and examined for β -galactosidase 15 activity, represented by a blue coloured cell cytoplasm.

2. Cell kinetics of alginate encapsulated cells

The *in vitro* cell cycle distribution of the 20 encapsulated NIH 3T3 cells was determined by flow cytometric DNA analysis. The encapsulated cells were released from the alginate by dissolving the beads in complete growth medium containing 1.5% tri-sodium citrate dihydrate (E. Merck) for 15 minutes, followed by 25 centrifugation at 140 g for 4 minutes, and removal of the supernatant. The cells were re-suspended twice in complete growth medium, centrifuged at 140 g for 4 minutes, fixed in ice cold 96% ethanol and stored at 4°C. Prior to the flow cytometric analysis, the cells were 30 incubated for 15 minutes with 0.5% pepsin (Sigma) in 0.9% physiological saline (pH = 1.5) at 37°C before the isolated nuclei were washed in 0.9% physiological saline,

and treated for 1 minute with ribonuclease (Sigma) (1 mg/ml in 0.9% physiological saline). Staining of DNA was obtained by adding propidium-iodide (Sigma) (50 μ g/ml in 0.9% physiological saline) to the nuclei. The cellular DNA content was measured using a Becton Dickinson FACSort flow cytometer (Becton Dickinson, Palo Alto, CA). The DNA histograms were obtained by gating a two parameter forward- and side-scatter cytogram to a one parameter DNA histogram. Each histogram was obtained by counting a total of 5000 gated nuclei. The flow-cytometric experiments were repeated three times, and the cell cycle distribution was determined as described in Radiat Environ Biophys, 12 (1975), pages 31-39.

3. Antibody release from the encapsulated hybridoma cells

Alginate beads with diameters between 2.3 and 2.5 mm containing 1.5×10^3 H528 cells per bead on the day of encapsulation were prepared as described above. After 0, 1, 5, 12, 19, 23, 30 and 33 days, respectively, beads were removed from the stock culture and the release of Mabs into the RPMI medium was examined. The beads were transferred into 24 well dishes (Nunc), in 0.5 ml complete RPMI medium (37°C). After 6 hours of incubation, four samples of 100 μ l each were collected, placed in 1.5 ml centrifuge test tubes (Treff AG, Degersheim, Switzerland) and frozen at -20°C).

Flow cytometry was used to determine the concentration of MAb in the samples. GaMg monolayer cell cultures were trypsinized with 2 mM EDTA in DPBS. The cells were then centrifuged at 140 g for 4 minutes, the

supernatant was removed, and the cells were fixed in 2% paraformaldehyde solution in DPBS for 1 minute. Thereafter the cells were centrifuged at 140 g for 4 minutes, and the supernatant was removed. The cells were then re-suspended in DPBS containing 2 mM EDTA, 1% bovine serum albumine and 1 g/l glucose, and distributed in a conical 96 well plate (Nunc) with 1.7×10^5 cells/well. The cells were centrifuged at 340 g for 4 minutes, and the supernatant was removed. Thereafter the cells were vortexed and incubated for 2 hours at 4°C with the harvested MAb RPMI medium (undiluted, and 1:5, 1:20 and 1:100 dilutions in DPBS). As a reference, an EGFR MAb (528) antibody (Santa Cruz Biotechnology, Santa Cruz, CA) with a known MAb concentration was used (concentrations 20, 5, 1, 0.2, 0.1 and 0.05 µg/ml). The cells were washed twice in 2 mM EDTA, 1% BSA, 1 g/l glucose in DPBS, and then incubated with FITC-conjugated goat anti-mouse immunoglobulins (Dako A/S, Glostrup, Denmark) (1:20 dilution) for 30 minutes at 4°C. Flow cytometry was performed on a Becton Dickinson FACSort flow cytometer. Single cells were detected and visualized by a two parameter forward- and side-scatter cytogram and gated to a one parameter FITC histogram, where the fluorescence intensity was determined. By using the various titers of the EGFR MAb with a known concentration on the GaMg cells, a reference antibody binding curve to GaMg cells was obtained. By comparing the results obtained from medium harvested from the hybridoma containing alginate beads, the MAb concentration curve was obtained.

4. Cell migration

GaMg spheroids were individually transferred to 16-mm multiwell dishes (Nunc), in 1.0 ml complete RPMI medium containing 10 ng/ml EGF (Sigma). Thereafter, the tumour cells were exposed to alginate beads containing H528 cells (three alginate beads in each well). As controls, spheroids were exposed to complete RPMI medium with or without 10 ng/ml EGF. The orthogonal diameter of each colony was measured daily for four days, using a light microscope with a calibrated reticle in the ocular.

The circular area covered by the cells migrating out from the spheroids was then determined and used as an index of cell migration. The experiments were performed in duplicate, with six spheroids in each experiment.

5. Establishment of endostatin producer cells and proof: The endostatin release from the beads

5A. Establishment of endostatin producer cells

Methods:

Cell line and culture conditions.

Human foetal kidney 293 cells (293-EBNA) expressing the Epstein-Barr virus nuclear antigen (EBNA) -1 were used as a producer cell line.

The cells were transfected with the episomal expression vector pCEP-Pu containing the gene encoding human endostatin, by liposomal and selected with 0.5 µg/ml puromycin.

The transfected cells (293-endo) were grown to confluency in 175 cm² culture flasks (Nunc, Roskilde, Denmark)

containing growth medium consisting of Dulbecco's modified Eagles medium (DMEM) supplemented with 10% heat-inactivated foetal calf serum, 4.5 g/l D-glucose, penicillin (100 IU/ml) and streptomycin (100 µl/ml), 205 5 µg/ml geneticin (G-418) and 0.5 µg/ml puromycin. Mock transfectants were generated by transfecting 293 cells with the pCEP-Pu vector without the endostatin gene and grown under the same conditions with the exception of puromycin (all Biochemical products from Biowhitaker, 10 Verviers, Belgium).

The tumour cell line (BT4C) chosen for these experiments was developed from an ethylnitrosourea induced rat gliosarcoma (passage number 26) and is syngeneic in BD- 15 IX. The cells were grown to confluency in 80 cm² culture flasks with complete growth medium consisting of Dulbecco's modified Eagles medium (DMEM) supplemented with 10% heat-inactivated new-born calf serum, 4 times the prescribed concentration of non-essential amino 20 acids, 2% L-Glutamin, penicillin (100 IU/ml) and streptomycin (100 µl/ml).

5b. **Estimation of endostatin release from the beads.**

25 **Immunoblots.**

Conditioned medium from encapsulated endo-293 and 293-EBNA was collected and used for standard SDS/PAGE Western blotting to determine whether endostatin was released from the beads.

30 Briefly, the samples were separated on a 12% SDS gel and blotted onto a PVDP nitrocellulose membrane. The blots were washed with 100% methanol for 5 min, dest. water 1

min, blocking solution (0,05 M Tris/HCL, 0.45 M NaCl, 2% Tween ,ph 10.2) 4 min and finally with washing buffer (0,05 M Tris/HCL, 0.15 M NaCl, 0.05% Tween 20, ph 10.2.) for 15 min. The blots were then incubated over night with
5 Rabbit anti-human anti-sera (1:1000 in wash buffer).
Following over night incubation, the blots were washed in DPBS and incubated with pig anti-rabbit alkaline phosphatase conjugated IgG (DAKO, Denmark).
Visualisation of the bands was performed by incubation
10 with of the substrate staining solution (2-4 min).

IN VIVO EXPERIMENTS

15 1. Intracranial implantations

Male inbred BD-IX rats (36) weighing between 160 g and 250 g were kept on a standard pellet diet, given unlimited access to tap water and caged
20 individually at a constant temperature and humidity on a 12 hour light and dark schedule. The rats were anaesthetized intraperitoneally with pentobarbitol at a concentration of 0.4 ml/100 g body weight. Via a mid-sagittal skin incision, a burrhole was made with a 3.5 mm
25 drill 4.2 mm posterior to the bregma point and 2.5 mm to the right of the sagittal suture. Cortical and white matter tissue was removed by suction to a depth of 2.0 mm, and between 8 and 14 alginate beads (one day old beads) containing either NIH 3T3 cells or H528 cells were
30 placed in the tissue cavity. The burrhole was closed with bone wax and the skin sutured with polyamide thread. Recovery under a heating lamp was allowed for 1 hour.

The animal care was in accordance with institutional guidelines. The rats were observed once a day, and weighed every other day. All animals recovered quickly after the implantations, and did not show any signs of illnesses or neurological deficits during the observation period.

2. Release and dissemination of immunoglobulins within the rat brain

10

After 3 and 9 weeks, the rats were sacrificed by CO₂ inhalation. The brains were removed, embedded in Tissue Tek (Miles Laboratories Inc., Naperville, IL) and frozen in 2-Methylbutane (E. Merck) cooled with liquid nitrogen. Axial sections (14 μ m) were cut on a Reichert-Jung cryocut 1800 cryotome (Leica, Wetzlar, Germany), and stored at -20°C. Cryosections obtained from rats implanted with H528 encapsulated cells and sacrificed after 3 weeks, were fixed in acetone for 5 minutes at room temperature, and then washed twice in DPBS for 5 minutes. The sections were then incubated with FITC-conjugated goat anti-mouse immunoglobulins (Dako A/S; 1:20 dilution) for 1 hour at room temperature, and thereafter washed for 5 minutes with DPBS. The sections were treated for 30 seconds with ribonuclease (Sigma) (0.5 mg/ml in 0.9% physiological saline), and staining of the nuclei was obtained by adding propidium-iodide (Sigma) (50 μ g/ml in 0.9% physiological saline) to the sections. Furthermore, the sections were washed with DPBS for 5 minutes, and then mounted with Vectashield (Vector Laboratories Inc, Burlingame, CA). The fluorescence was measured using a Leica TCS NT confocal laser scanning

microscope with an argon-krypton laser (Leica), using TRITC and FITC filter optics. Sections taken from the same depth within the brains of the experimental animals were investigated, and the areas of maximum fluorescence intensity were studied in both groups. Cryosections obtained from rats implanted with NIH 3T3 cells and sacrificed after 9 weeks, were stained with Haematoxylin and Eosin for histological examination.

10

3. Immune responses towards producer cells encapsulated in alginate.

Methods

15 The percentage of immuno-positive cells at the border zone between brain to BD-IX rats and alginate beads was evaluated 1,3 and 9 weeks after implantation. The brains were mounted on stubs, embedded in tissue-tek and frozen in liquid N₂. Serial axial sections 5-10 µm were cut on a
20 Reichert Jung Cryostat (Leica, Wetzlar, Germany) mounted on slides and prepared for immunohistochemical analyses. Sections were fixed in cold acetone for 5 min, incubated for 30 min. at room temperature with 10% normal rabbit Serum, diluted in PBS, and thereafter incubated over-
25 night at 4°C in a humidity chamber with mouse monoclonal antibodies (mAbs) diluted in 10% rabbit serum.

The following mAbs were used: OX42, ED1, and ED2 anti-rat macrophage mAbs, OX19 against CD5 positive T cells, and OX33 reactive with CD45RA positive B cells. The mAbs were
30 obtained from Serotec, Oxford, UK.

Biotinylated rabbit anti-mouse immunoglobulins diluted 1:300 were applied for 30 min. Avidin-biotin-peroxidase

complex (ABCcomplex/HRP, Dakopatts, Glostrup, Denmark) was prepared as recommended by the manufacturer, and allowed to react with the sections for 30 min. Finally, the sections were treated with a buffer containing 3-
5 amino-9-ethyl-carbazole, for the development of a coloured reaction product. Washing in PBS followed all incubations. All preparations were counterstained with hematoxylin, mounted in Glycergel (Dakopatts,), and analysed by light microscopy.

10

4. Effects of endostatin alginate-therapy on tumour growth

Young adult BD-IX rats of both sexes (8 rats in total,
15 plus 20 controls) were anaesthetised by intraperitoneal injections of Equithesine at a dosage of 0.4 ml/100 g body weight. The rats were immobilised in a stereotactic frame (David Kopf Instruments, Tujunga, USA), the skin was incised and a 2 mm burr hole was made 1 mm posterior
20 to and 3.0 mm to the right of the Bregma point, and inserted to a depth of 2.5 mm. Using a injected into the brain. Following this 1x10⁴ BT4C gliosarcoma cells were injected 1 mm lateral to the alginate beads at a depth of 2mm. The alginate beads contained either endostatin
25 producing 293 cells or 293-mock transfectants, as controls. Eight animals received implants form each cell line. Furthermore, as a control of normal tumour progression, 8 animals were injected with BT4C cells alone.

30 Finally, as a control of the *in vivo* viability of the cells within the beads, the remaining 4 control animals received alginate beads containing 293-endo cells alone.

The syringe was slowly retracted over 3 min (for all injections) and closure was performed with bone wax and suture. The animals were allowed to recover from surgery under observation. During the experimental period the 5 animals were housed in pairs at constant temperature and humidity, fed a standard pellet diet and provided tap water *at libitum*.

RESULTS

10

IN VITRO EXPERIMENTS

1. Morphology and viability of the alginate encapsulated cells

15

Alginate beads with diameters of 1.0 mm contained approximately 6.5×10^2 NIH 3T3 cells on the day of encapsulation (Fig. 1A). The cells were evenly distributed within the alginate beads, with an outer cell 20 free rim of 25-50 μm . During culture, cell proliferation was observed within the alginate, resulting in an increased cellular density after 3 weeks (Fig. 1B). After 9 weeks in culture, multicellular spheroids were observed within the alginate beads (Fig. 1C). Over 90% of the 25 beads remained intact after 9 weeks in culture, as assessed by light microscopy. After about a week in culture a few single cells migrated out from the alginate beads and into the growth medium, and this limited movement of single cells continued during the next 8 30 weeks of culture.

The confocal laser scanning microscopy study showed that around 90% of the encapsulated cells remained viable on the day of encapsulation (Fig. 1D). After 3 weeks in culture, around 50% of the originally
5 encapsulated cells were viable (Fig. 1E). Some of the surviving cells adapted to the alginate and formed viable multicellular spheroids, which could be clearly observed after 9 weeks (Fig. 1F). At this time point the total number of viable cells within the beads were difficult to
10 assess due to the multicellular spheroid formation. However, as shown in Fig. 1F, most of the cells localized in the spheroids were viable.

The encapsulated BT4CnVlacZ cells expressed a
15 constant and evenly distributed β -galactosidase activity during the whole observation period of 9 weeks (Fig. 1G).

2. Cell kinetics of alginate encapsulated cells

20 The flow cytometric histograms of the NIH 3T3 cells showed a change in cellular ploidy within the alginate beads 1 week after encapsulation (Fig. 2B). This probably represents a polyploidization, as compared to the diploid control (Fig. 2A). However, after 3 and 9
25 weeks respectively (Fig. 2C, 2D) a normalization in ploidy was observed, with a similar diploid distribution as for the controls. The fraction of proliferating cells in the S and G₂M phases was 50% for the control, as compared to 55% and 60% after 3 and 9 weeks *in vitro*,
30 respectively.

3. Antibody release from the encapsulated hybridoma cells

Already at the end of the first day of encapsulation, there was a release of 13 ng/(ml*hr) of
5 MAbs in the growth medium (Fig. 3). The diffusion of immunoglobulins out of the beads and into the medium increased steadily during the next days of culture, and reached a concentration of 457 ng/(ml*hr) after 12 days. The production of MAbs then stabilized around
10 400 ng/(ml*hr) during the last 3 weeks of the observation period.

4. Cell migration

15 The migration of cells out from the GaMg spheroids stimulated with EGF was extensive, and the mean outgrowth area was doubled, compared to the controls (Fig.4). However, when alginate beads containing H528 cells were added in the presence of EGF, cell migration
20 was strongly inhibited, demonstrating that the encapsulated H528 producer cells effectively express an antibody directed against the EGF receptor.

5. Estimation of endostatin release from the beads.

25

As seen from the western blots of conditioned medium harvested from the beads, a substantial amount of endostatin is released from the beads (Figure 6). The radioimmunoassays have shown that 10 endostatin producing
30 alginate beads (400 µm) with 25000 cells encapsulated, secreted 2.5 µg/ml/24 hrs.

IN VIVO EXPERIMENTS

1. Intracranial implantations

5 Axial sections of the rat brains revealed little or no change in the brain parenchyma adjacent to the implantation site harbouring the alginate encapsulated NIH 3T3 cells (Fig. 5A). Little intracranial edema or swelling was observed after 9 weeks. The
10 alginate beads were free of any cell overgrowth, and contained both viable single cells and multicellular spheroids (Fig. 5B). The viable cells were distributed both in the centre and in the periphery of the beads, with cell-free areas of alginate in between the cells. A
15 minimal aggregation of cells around the border zone between the implantation hole and the brain parenchyma was observed.

2. Release and dissemination of immunoglobulins within 20 the rat brain

 The implanted beads with encapsulated hybridoma cells were easily visualized after 3 weeks by an intense green fluorescence (Fig. 5C). Immunoglobulins were
25 detectable in the brain tissue at a distance of at least 1 mm from the alginate beads (Figs. 5C, 5D), with a gradual decrease in fluorescence intensity from the border of the implantation site and into the brain. For two of the experimental animals, MAbs were detected in
30 the whole cerebral hemisphere, where the implants were located (data not shown). MAbs were further found in the leptomeninges in both hemispheres of the cerebrum

(Fig.5E), with the strongest fluorescence seen in the subarachnoidal area in the right hemisphere. The negative controls showed a weak fluorescence in the leptomeninges, probably caused by non-specific binding between the immunoglobulins and epitopes on the leptomeningeal cells (Fig.5F). However, the brain parenchyma was negative. The MABs were further present in the perivascular space of intracerebral blood vessels, with no apparent difference in fluorescence intensity between the two hemispheres (Fig.5G). The weak fluorescence present in the control was again probably caused by non-specific binding (Fig.5H).

3. Immune responses towards producer cells encapsulated in alginate.

Infiltration of mononuclear cells was observed in the brain adjacent to the alginate beads. The amount of cells in the infiltrate decreased from week 1 to week 9. One week after implantation OX42 positive microglia with a dendritic morphology were seen in the parenchyma and reactive microglia and invading monocytes appeared at the border zone towards the alginate beads. ED1 and ED2 stained monocytes close to the border zone whereas few cells were stained by these mAbs elsewhere in the brain parenchyma. A limited number of T and B cells were also observed at the border zone to the beads (Table I). The amount of OX42 positive cells at the border zone decreased from 62% at week one to 20% at week 9, whereas the ED1 positive cells decreased from 34% at week one to 7% at week 9. The amount of ED2 positive cells (5%), T cells (14%), and B cells (1%) changed only marginally

during the observation period (Table I).

Table I

**5 Cellular immune response in rat brain tissue 9 weeks
after implantation of NIH 3T3 cells in alginate**

<i>Cells</i>	<i>Immunoreactivity</i>
T cells (CD5)	No reactivity
10 B cells (CD45RA)	No ractivity
Microglia and macrophages (OX42)	High reactivity
Macrophages and monocytes (ED1/ED2)	Weak reactivity

**15 4. Effects of endostatin alginate-therapy on tumour
growth**

The animals treated with endostatin-producer cells in
alginate lived 20% +/- 4% longer than animals treated
20 with mock transfected cells. Detailed histological
observations revealed large necrotic areas in the tumours
that received endostatin-alginate therapy (see Figure 7,
panel B). Such necrotic areas were never seen in the
controls (mock transfected cells encapsulated in
25 alginate; Figure 7, panel A).

DISCUSSION

The results of the above-described experiments
30 clearly demonstrate that the microencapsulated cells
survive, proliferate and maintain their phenotypic
expression over extended time periods. It is also shown
that MAbs released from the alginate beads have the

ability to inhibit tumour cell migration *in vitro* by interfering with the EGFR, and that MAbs are released and disseminated within the rat brain.

5 As seen by light microscopy, the NIH 3T3 cells adapted to the alginate *in vitro*, and started to proliferate within a few days after encapsulation. The CLSM study revealed cell viability around 90% at the day of encapsulation. During the first three weeks in
10 culture, around 50% of the initially entrapped cells died within the beads. However, after 9 weeks, the remaining cells showed the ability to form multicellular spheroids within the alginate. An observed cell death within the alginate has also been reported by others, and may be due
15 to a reduced diffusion of oxygen, nutrients and waste products, which may eventually lead to an equilibrium between the number of proliferating and dying cells. A more favourable diffusion rate may be achieved by decreasing bead size, increasing the content of G-units,
20 which would increase the pore sizes, or changing the alginate concentration. In addition, the diffusion is dependent on the number of initially encapsulated cells within the beads. The alginate itself is non-toxic, and can therefore not be expected to contribute to the
25 observed cell death within the beads.

The BT4CnVlacZ cells exhibited a strong and evenly distributed β -galactosidase activity during 9 weeks of culture. These results demonstrate that also
30 specific gene products may be produced during prolonged periods within alginate beads.

The flow cytometric study showed that the NIH 3T3 cells changed from a diploid to a multiploid population after 1 week in alginate. This indicates that the cell nuclei divide, but because of limited space within the rigid alginate network, the cells are initially not able to undergo cytokinesis. This will then result in single cells with double and triple nuclei (Fig. 2B). However after 3 weeks, the cell cycle distribution was similar to the controls. This may indicate that the cells need a certain adaptation period within the alginate, where single cells with double and triple nuclei will either finish their cytokinesis or die. The histograms after 9 weeks were similar to those after 3 weeks, but indicated an increase of cells in the proliferating phases. The analysis of the cell cycle distribution showed an increase in the number of proliferating cells, from 50% for the control, to about 60% after 9 weeks. This may be due to a selection within the alginate beads of cells with a higher proliferative capacity during prolonged culture of the NIH 3T3 cells.

The antibody release from the encapsulated H528 hybridoma cells was substantially constant at around 400 ng/ml*hr from day 12 to day 33, which shows that a stable density of MAb-secreting hybridoma cells had been established after 12 days in culture. This finding is important for the clinical situation, as it shows stable monoclonal antibody production is achieved at a high level.

The cell migration out from the GaMg spheroids was stimulated in the presence of EGF. By adding H528 encapsulated cells to the EGF stimulated spheroids, the migration was inhibited, and the outgrowth area was
5 similar to the controls. This implies that paracrine cell proliferation mechanisms are inhibited by these Mabs, probably by blocking the EGF-binding domain of the EGFR.

Implantation of alginate-encapsulated producer
10 cells in other organs outside the central nervous system (CNS) has shown a fibroblast overgrowth of the alginate beads, leading to cell death and graft failure (Transplantation, 54 (1992), pages 769-774). Due to the unique location, and the lack of fibroblasts in the CNS,
15 the same cell overgrowth was not observed in the present study (Fig. 5A, B). Depending on the composition, alginates have in some instances been shown to trigger an immune response within the body by stimulating monocytes to produce high levels of cytokines. The cytokine-
20 stimulating part of the alginate are the M-units. An alginate with a high content of G-units was therefore chosen for our experiments, in order to minimize the immune response within the brain. In further experiments we have found a low immune response towards alginate
25 encapsulated cells within the brain, with only some microglial cells assembling in the brain tissue close to the implanted beads. These observations further show alginate-encapsulated producer cells to be an attractive treatment within the brain. A minimal aggregation of
30 cells around the border zone between the implantation site and the brain parenchyma was also seen. This may be due to NIH 3T3 cells escaping from the alginate beads,

because of a mild immune response towards the implants as discussed above, and/or due to a tissue wound-healing process. It is, however, not considered that the small number of producer cells which escape the alginate
5 represents a problem, since these cells would be taken care of by normal graft versus host rejection mechanisms. However, if desired, steps can be taken to prevent cell escape eg by covering the beads with a layer of poly-L-lysine or by irradiating the cells prior to
10 encapsulation, thereby inhibiting their proliferative capacity. The immunoglobulins were released from the alginate beads, and disseminated into the brain parenchyma at a distance of at least 1 mm away from the border of the implantation site. In two of the
15 experimental animals, MAbs were also detected in the whole cerebral hemisphere where the implants were located. This dissemination may be due to a passive diffusion process. MAbs were also localized in the subarachnoidal area and within the perivascular space of
20 Virchow-Robin. This spread is most likely mediated by the constant flow of cerebrospinal fluid within the CNS. Interestingly, tumour cells follow the same dissemination pathways within the brain, which make them accessible to components produced by alginate encapsulated cells.

25

In summary, the experiments which are described above show that encapsulated producer cells survive and proliferate within the alginate for prolonged time periods, *in vitro* as well as *in vivo*. Gene products such
30 as β -galactosidase are produced within the cell cytoplasm of the encapsulated BT4CnVlacZ cells during several weeks of culture. Encapsulated hybridoma cells further produce

and release high amounts of MAbs *in vitro* and *in vivo*.
The GaMg tumour cell migration is inhibited in the
presence of encapsulated H528 cells. Implants of
encapsulated H528 cells also produce and release MAbs
5 within the rat brain, and the MAbs disseminate within the
brain parenchyma, as well as within the subarachnoidal
and in the perivascular space. The present invention
therefore represents a promising tool for CNS tumour
therapy.

CLAIMS

1. An encapsulated producer cell capable of expressing a molecule which is an inhibitor of CNS tumour growth, wherein the encapsulation matrix is an immuno-isolating alginate.

2. An encapsulated producer cell according to Claim 1, wherein said molecule is a protein or peptide capable of interacting with tumour/host communication pathways.

3. An encapsulated producer cell according to Claim 2, wherein said protein or peptide is capable of affecting tumour neovascularization.

4. An encapsulated producer cell according to Claim 2, wherein said protein or peptide is capable of interfering with the relationship of the tumour cell with the extracellular matrix.

5. An encapsulated producer cell according to Claim 1, wherein said molecule is a protein or peptide capable of interacting directly with the tumour cell.

6. An encapsulated producer cell according to Claim 5, wherein said producer cell is a hybridoma cell capable of expressing a monoclonal antibody capable of interacting directly with a receptor of the tumour.

7. An encapsulated producer cell according to any preceding claim, wherein said expression of said molecule is capable of being switched on and off by an external pharmacological agent.

8. An encapsulated producer cell according to claim 1, wherein the alginate has a G content of above 15%, more preferred above 50%.

9. An encapsulated producer cell according to Claim 8, wherein the alginate has a G content of 60%-80%.

10. An encapsulated producer cell according to Claim 1, wherein the alginate has a G content of 80%-100%.

11. An encapsulated producer cell according to any one of Claim 1 in the form of beads or microbeads.

12. An encapsulated producer cell according to any preceding claim, wherein said tumour is a brain tumour.

13. A method for the treatment of CNS tumour, which comprises implanting at the site of the tumour an immuno-isolating alginate encapsulated producer cell which is capable of expressing a molecule which is an inhibitor of the growth of said tumour.

14. A method according to Claim 13, wherein said encapsulated producer cell is as defined in any one of Claims 1-12.

15. A method according to Claim 13 or Claim 14, wherein said producer cells are implanted following surgical removal of said tumour.

16. A method for the preparation a pharmacological product for the treatment of a CNS tumour, which comprises encapsulating within an immuno-isolating matrix a producer cell capable of expressing a molecule which is an inhibitor of the growth of said tumour.

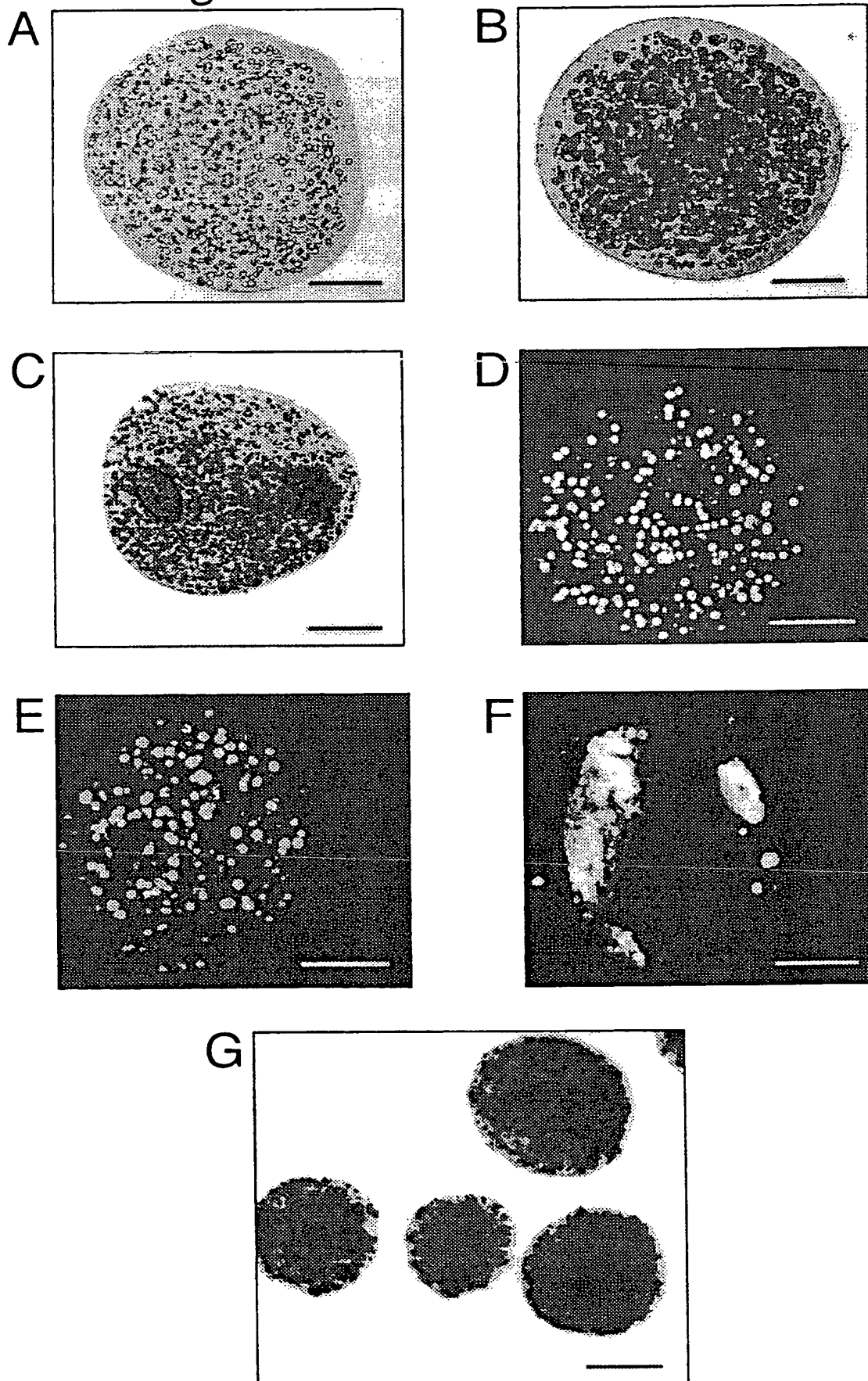
17. A use of an immunoisolating alginate for preparing encapsulated producer cells intended for transplantation in the brain after surgical removal of a brain tumour, which producer cell capable of expressing a molecule which is an inhibitor of the growth of said tumour.

18 A use according to claim 17, wherein the alginate is of a high-purity, endotox in free quality.

19. A use according to claim 17, wherein the alginate has a G content of at least 15%, more preferred above 50%.

Fig.1.

1/7



2/7

Fig.2.

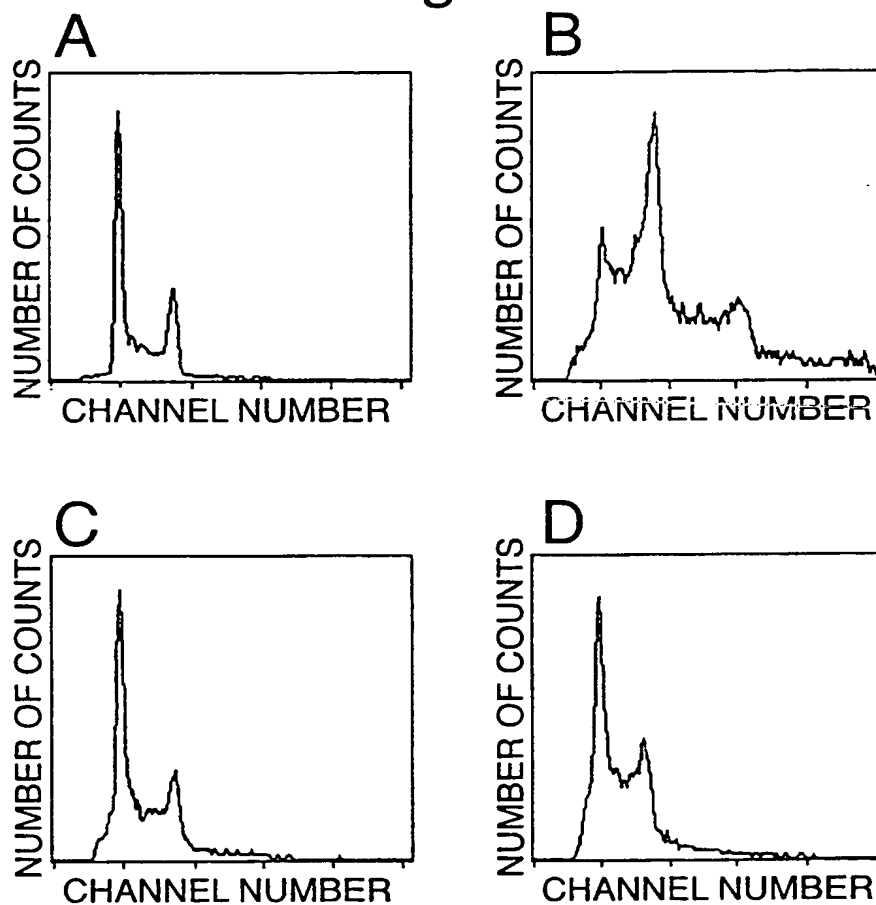
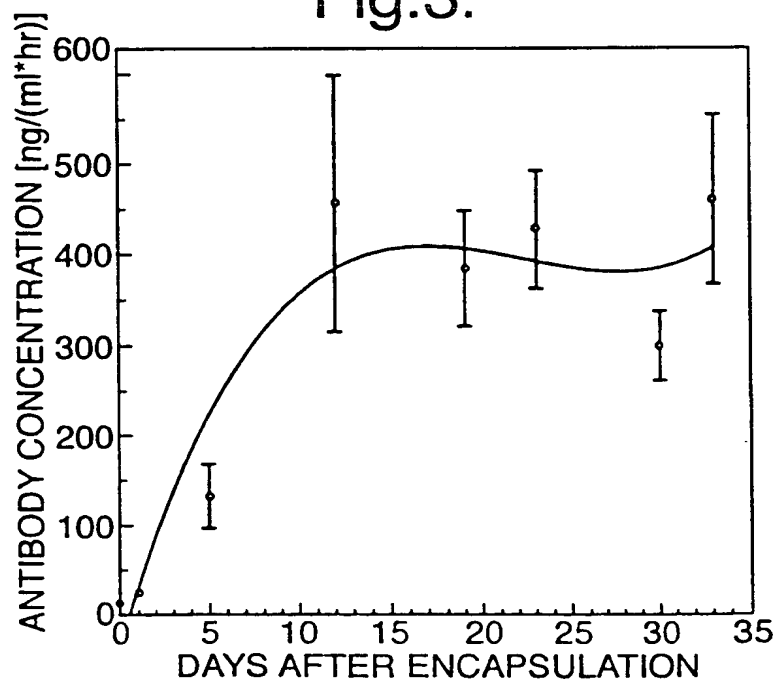


Fig.3.



3/7

Fig.4.

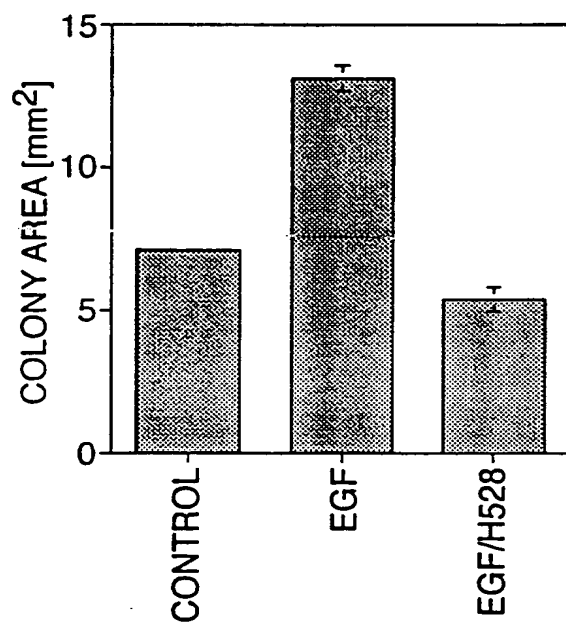


Fig.5.

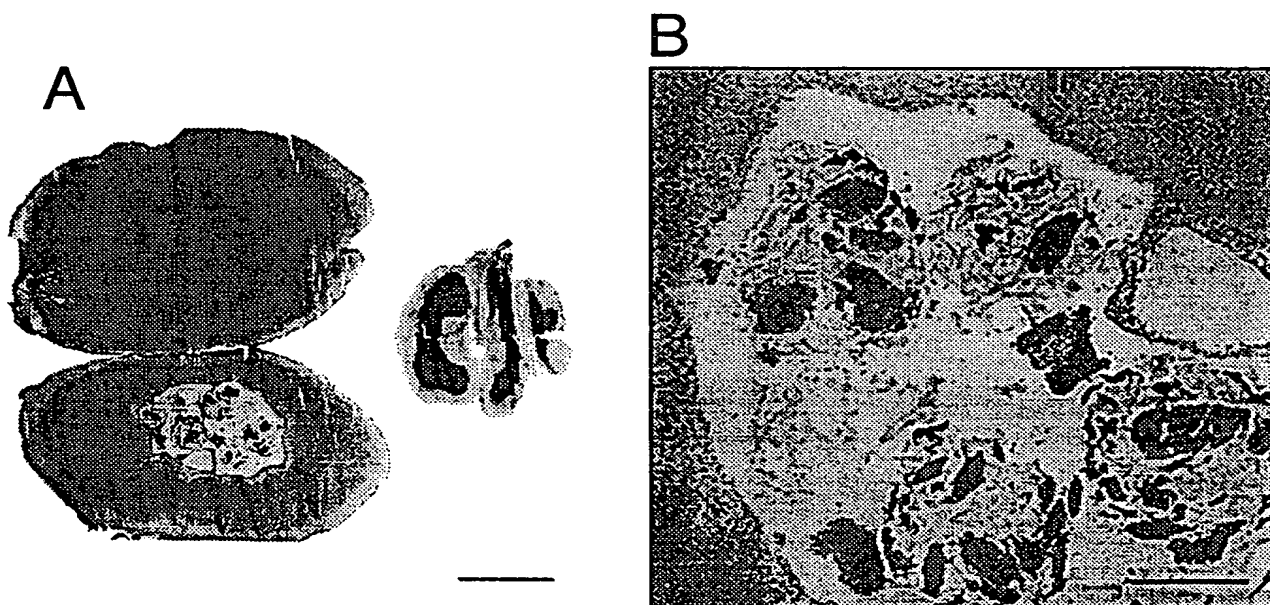


Fig.5 (Cont i).

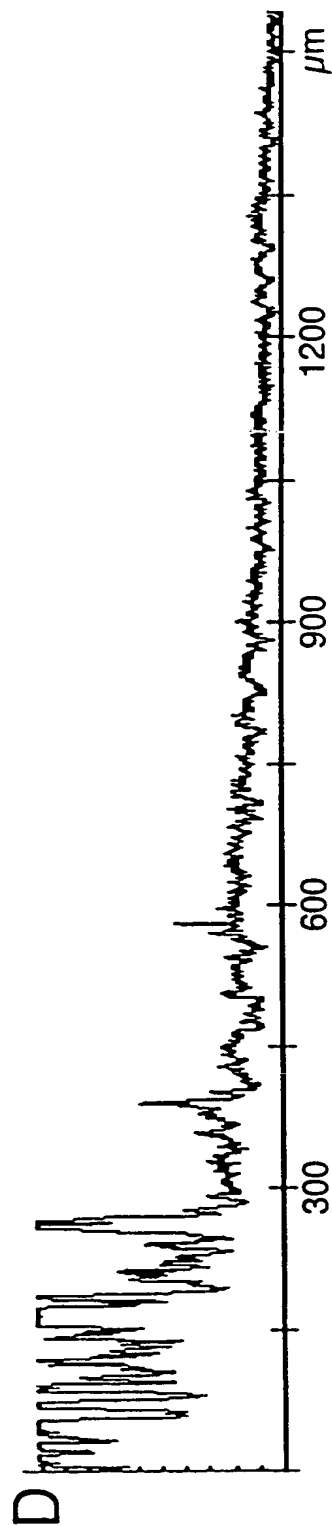
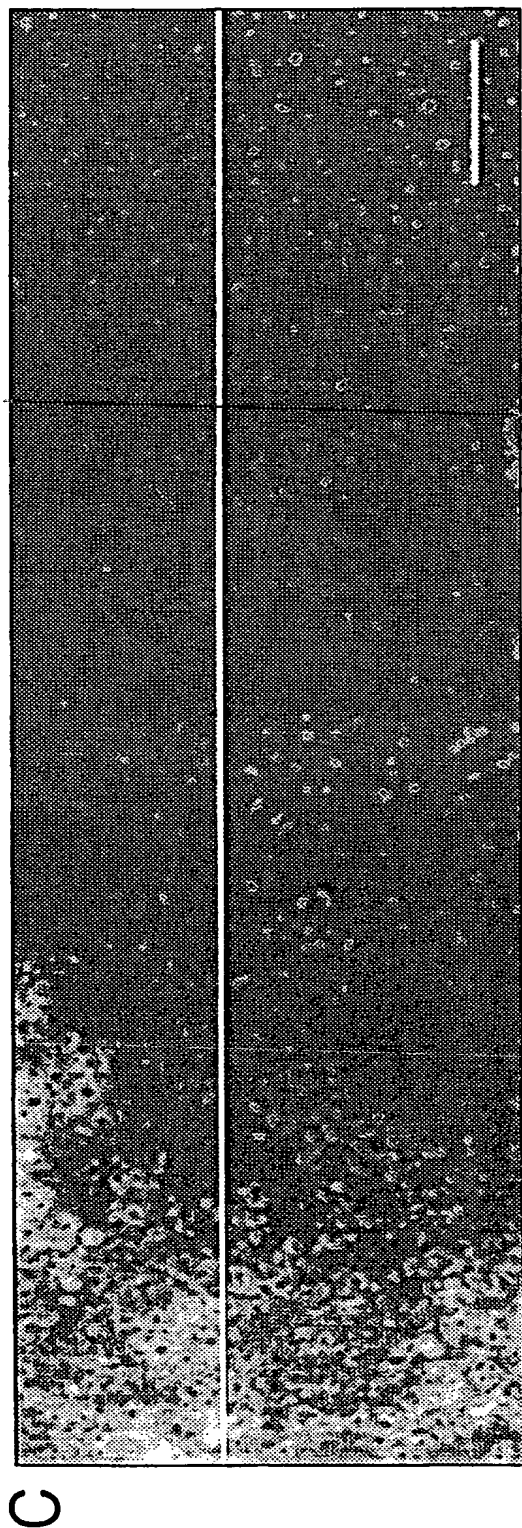


Fig.5 (Cont ii).

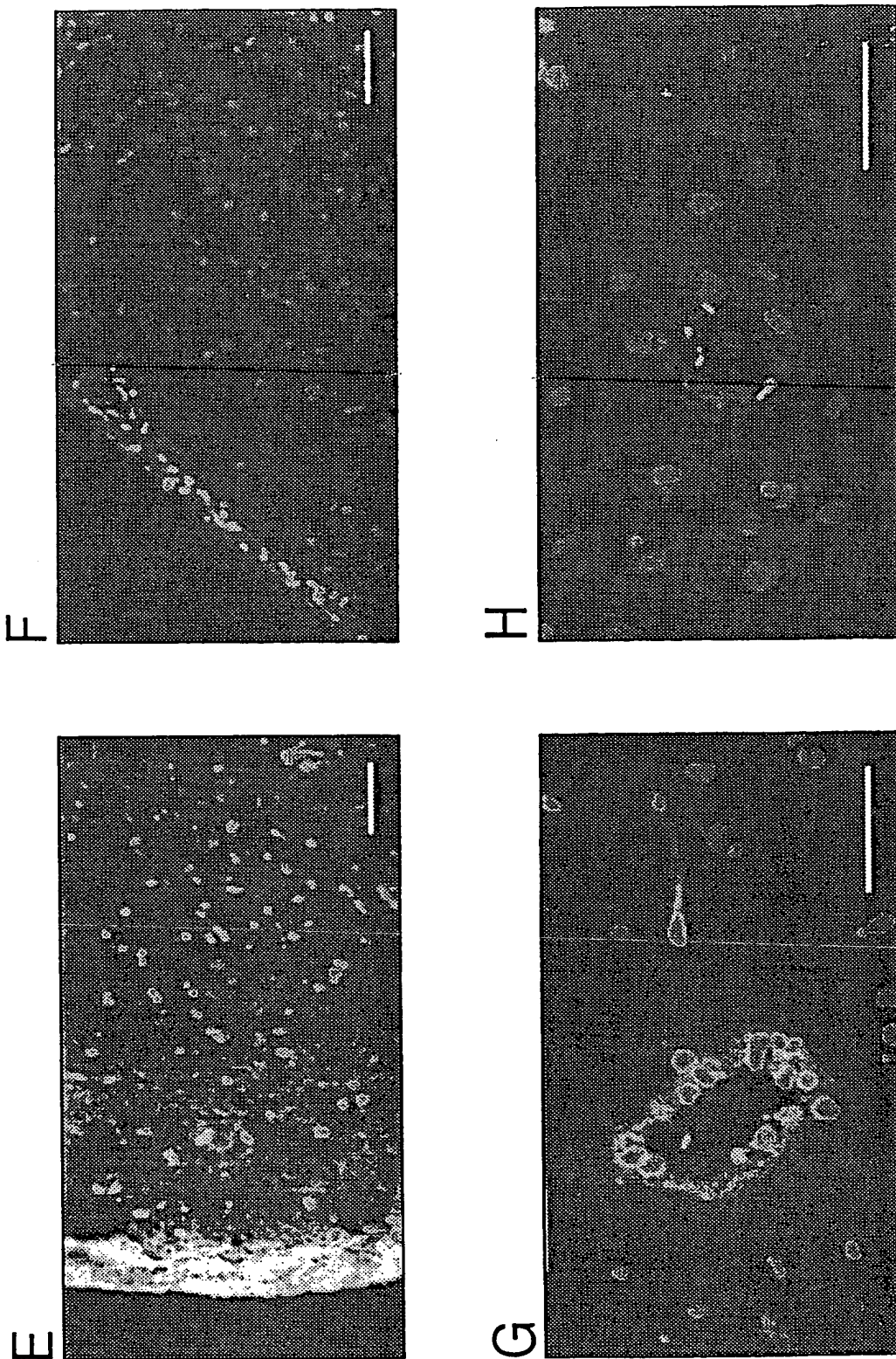


Fig. 6.

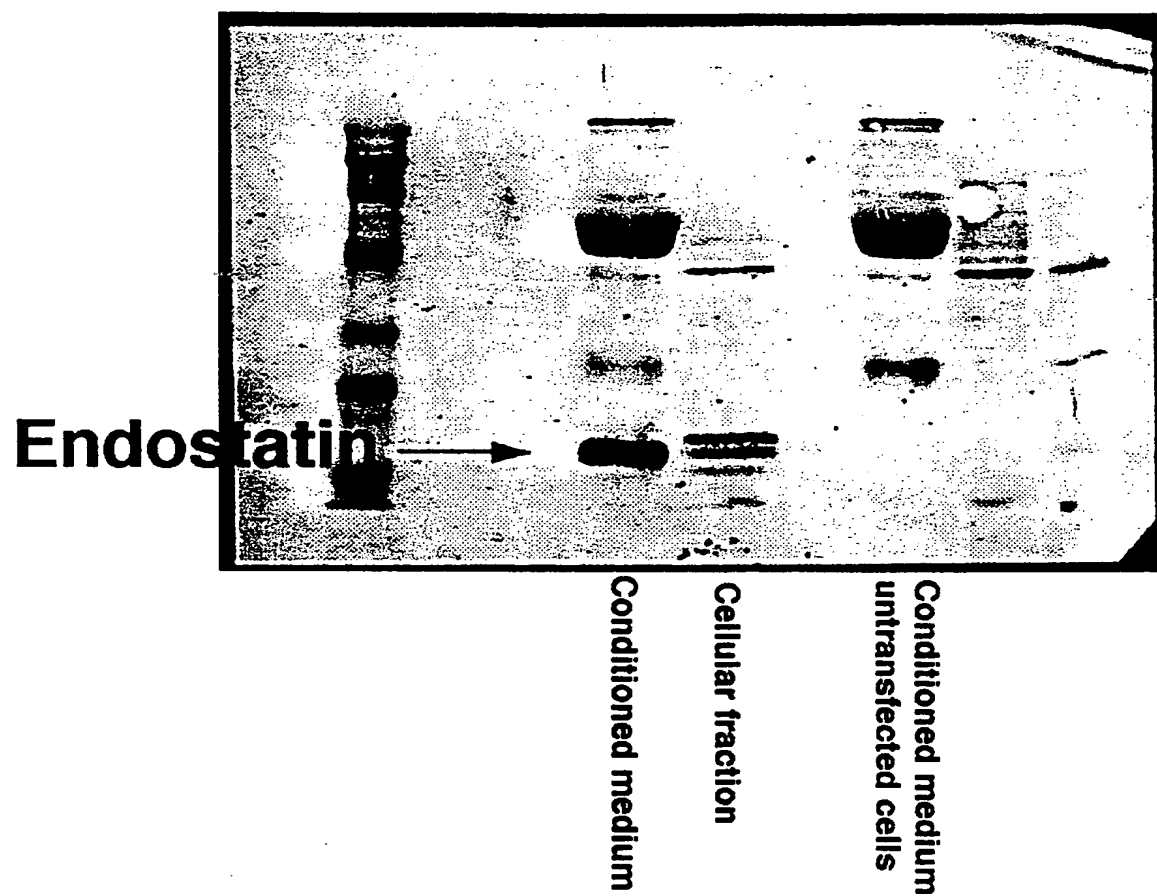
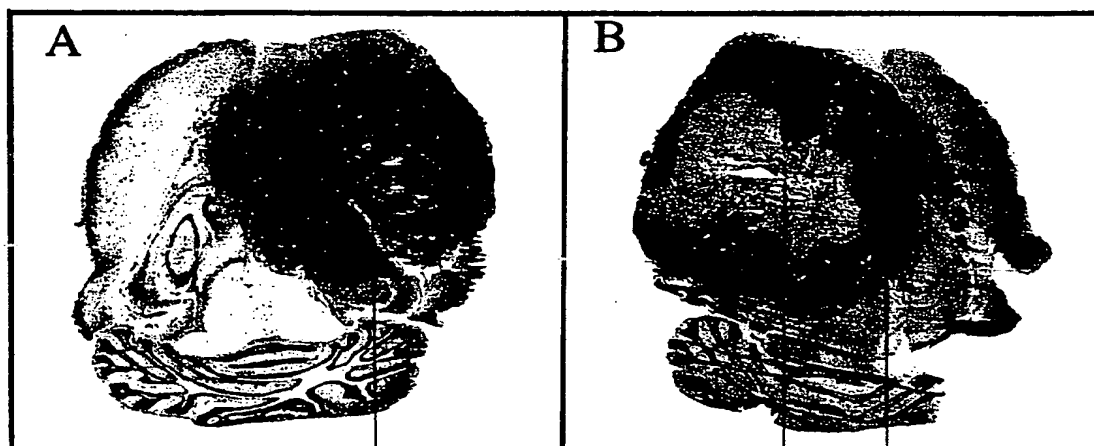


Fig. 7.



Tumour

Tumour

Necrotic area in the
tumour

INTERNATIONAL SEARCH REPORT

International application No.

PCT/NO 99/00266

A. CLASSIFICATION OF SUBJECT MATTER

IPC7: A61K 9/16, A61P 35/00 // C12N 11/04

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC7: A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	INT. J. DEV. NEUROSCI, Volume 17, No 5-6, 1999, Tracy-Ann Read et al, "Cells encapsulated in alginate: a potential system for delivery of recombinant proteins to malignant brain tumours" page 653 - page 663 --	1-19
X	WO 9738707 A1 (CYTOTHERAPEUTICS, INC.), 23 October 1997 (23.10.97), page 2, line 22 - page 3, line 11; page 15, line 9 - line 30; page 16, line 10 - page 17, line 17, page 10, line 24 - line 27; page 19 --	1-19
A	WO 9628029 A1 (BIOHYBRID TECHNOLOGIES, INC), 19 Sept 1996 (19.09.96) --	1-19

☒ Further documents are listed in the continuation of Box C.☒ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

30 November 1999

Date of mailing of the international search report

13 -01- 2000

Name and mailing address of the ISA/

Swedish Patent Office

Box 5055, S-102 42 STOCKHOLM

Facsimile No. +46 8 666 02 86

Authorized officer

Hampus Rystedt/ELY

Telephone No. +46 8 782 25 00

INTERNATIONAL SEARCH REPORT

International application No.

PCT/NO 99/00266

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 9219195 A1 (BROWN UNIVERSITY RESEARCH FOUNDATION), 12 November 1992 (12.11.92) --	1-19
A	Acta Neurol Scand, Volume 97, No 1, January 1998, Lund EL et al, "Tumor angiogenesis - a new therapeutic target in gliomas", page 52 - page 62, abstract --	2-4
A	Anal Biochem, Volume 235, No 2, March 1996, Yin DX et al, "Tetracycline-controlled gene expression system achieves high-level and quantitative control of gene expression", page 195 - page 201, abstract --	7
A	J Immunother, Volume 10, No 4, August 1991, Otterlei M et al, "Induction of cytokine production from human monocytes stimulated with alginate", page 286 - page 291, abstract -- -----	8-10

INTERNATIONAL SEARCH REPORT

International application No.
PCT/NO99/00266

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 13-15
because they relate to subject matter not required to be searched by this Authority, namely:
Claims 13-15 relate to methods for therapeutic treatment of the human or animal body, see Rule. 39.1.(iv). Nevertheless, a search has been executed for these claims. The search has been based on the alleged effects of the compounds/compositions.
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

See extra sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☒ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

According to Article 34(3)(a-c) and Rule 13.2, an international application shall relate to one invention only or to a group of inventions linked by one or more of the same or corresponding "special technical features", i.e. features that define a contribution which each of the inventions makes over the prior art. All claims of the present application relate to alginate encapsulated cells producing tumour inhibitors. However, as this feature is not novel, see eg. WO-A1-9738707, the claims lack the "special technical feature" demanded by Rule 13.2 and the following inventions or groups of inventions were found:

1. Alginate encapsulated cells producing a tumour inhibiting substance, according to claims 1, 13 and 14 (partially)
2. Alginate encapsulated cells producing different tumour inhibitors, according to claims 2-5 and 14 (partially)
3. An alginate encapsulated hybridoma cell according to claim 6 and 14 (partially)
4. An alginate encapsulated cell in which the tumour inhibitor expression can be turned on or off, according to claims 7 and 14 (partially)
5. Cells encapsulated in various alginate compositions, according to claims 8-10 and 14 (partially), 18 (partially) and 19 (partially)
6. Microbeads of alginate encapsulated cells, according to claims 11 and 14 (partially)
7. Alginate encapsulated cells producing a brain tumour inhibitor, according to claims 12 and 14 (partially)
8. Implanting tumour inhibitor producing cells after surgical removal of a brain tumour, according to claim 15, 17, 18 (partially) and 19 (partially)

The characterising feature of invention 1 is the encapsulation of the cells in an immunoisolating alginate. This feature is known through the above mentioned WO-A1-9738707.

Invention 2 is characterised by the tumour inhibitors used.

Invention 3 is characterised by the cell type used.

Invention 4 is characterised by the possibility to turn the expression of tumour inhibitor on or off.

Invention 5 is characterised by the alginate compositions used.

Invention 6 is characterised by the physical form of the pharmaceutical product.

Invention 7 is characterised by the type of tumour targeted for inhibition.

Invention 8 is characterised by the method in which the encapsulated cells are used.

In summary, inventions 1-8 are not linked by a special technical feature as required by Rule 13.2 and the application therefore lacks unity of invention.

INTERNATIONAL SEARCH REPORT
Information on patent family members

02/11/99

International application No.

PCT/NO 99/00266

Patent document cited in search report.			Publication date	Patent family member(s)		Publication date
WO	9738707	A1	23/10/97	AU	2674097 A	07/11/97
WO	9628029	A1	19/09/96	AU	5186596 A	02/10/96
				CA	2215039 A	19/09/96
				EP	0813365 A	29/12/97
				JP	11501928 T	16/02/99
WO	9219195	A1	12/11/92	AT	156344 T	15/08/97
				AU	666118 B	01/02/96
				AU	682796 B	16/10/97
				AU	2004192 A	21/12/92
				AU	3902095 A	01/02/96
				DE	69221484 D, T	19/02/98
				DK	585368 T	16/03/98
				EP	0585368 A, B	09/03/94
				SE	0585368 T3	
				ES	2107537 T	01/12/97
				FI	934545 A	01/12/93
				GR	3025301 T	27/02/98
				HK	1001832 A	00/00/00
				JP	6507412 T	25/08/94
				NO	933842 A	25/10/93
				SG	47470 A	17/04/98
				US	5798113 A	25/08/98
				US	5800828 A	01/09/98
				US	5834001 A	10/11/98
				US	5869077 A	09/02/99
				US	5871767 A	16/02/99
				US	5874099 A	23/02/99

PCTWORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁷ : A61K 9/16, A61P 35/00 // C12N 11/04	A1	(11) International Publication Number: WO 00/12066 (43) International Publication Date: 9 March 2000 (09.03.00)
(21) International Application Number: PCT/NO99/00266 (22) International Filing Date: 25 August 1999 (25.08.99) (30) Priority Data: 19983911 26 August 1998 (26.08.98) NO (71) Applicant (for all designated States except US): NORSK HYDRO ASA [NO/NO]; N-0240 Oslo (NO). (72) Inventor; and (75) Inventor/Applicant (for US only): BJERKVIG, Rolf [NO/NO]; Skarehaugene 40B, N-5070 Mathopen (NO). (74) Agent: SANDBU, Elisabeth, Dahl; Norsk Hydro ASA, N-0240 Oslo (NO).		(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
(54) Title: ALGINATE CAPSULES FOR USE IN THE TREATMENT OF BRAIN TUMOUR (57) Abstract Encapsulated producer cells which are capable of expressing a molecule which is an inhibitor of CNS tumour growth provide a novel approach to the treatment of tumours, such as brain tumours which are localized within the central nervous system.		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece			TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon			PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

INTERNATIONAL SEARCH REPORT

International application No.

PCT/NO 99/00266

A. CLASSIFICATION OF SUBJECT MATTER

IPC7: A61K 9/16, A61P 35/00 // C12N 11/04

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC7: A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	INT. J. DEV. NEUROSCI, Volume 17, No 5-6, 1999, Tracy-Ann Read et al, "Cells encapsulated in alginate: a potential system for delivery of recombinant proteins to malignant brain tumours" page 653 - page 663 --	1-19
X	WO 9738707 A1 (CYTOTHERAPEUTICS, INC.), 23 October 1997 (23.10.97), page 2, line 22 - page 3, line 11; page 15, line 9 - line 30; page 16, line 10 - page 17, line 17, page 10, line 24 - line 27; page 19 --	1-19
A	WO 9628029 A1 (BIOHYBRID TECHNOLOGIES, INC), 19 Sept 1996 (19.09.96) --	1-19

☒ Further documents are listed in the continuation of Box C.☒ See patent family annex.

* Special categories of cited documents:

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

30 November 1999

Date of mailing of the international search report

13 -01- 2000

Name and mailing address of the ISA/

Swedish Patent Office

Box 5055, S-102 42 STOCKHOLM

Facsimile No. +46 8 666 02 86

Authorized officer

Hampus Rystedt/ELY

Telephone No. +46 8 782 25 00

INTERNATIONAL SEARCH REPORT

International application No.

PCT/NO 99/00266

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 9219195 A1 (BROWN UNIVERSITY RESEARCH FOUNDATION), 12 November 1992 (12.11.92) --	1-19
A	Acta Neurol Scand, Volume 97, No 1, January 1998, Lund EL et al, "Tumor angiogenesis - a new therapeutic target in gliomas", page 52 - page 62, abstract --	2-4
A	Anal Biochem, Volume 235, No 2, March 1996, Yin DX et al, "Tetracycline-controlled gene expression system achieves high-level and quantitative control of gene expression", page 195 - page 201, abstract --	7
A	J Immunother, Volume 10, No 4, August 1991, Otterlei M et al, "Induction of cytokine production from human monocytes stimulated with alginate", page 286 - page 291, abstract -- -----	8-10

INTERNATIONAL SEARCH REPORT

International application No.
PCT/NO99/00266

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 13-15
because they relate to subject matter not required to be searched by this Authority, namely:
Claims 13-15 relate to methods for therapeutic treatment of the human or animal body, see Rule. 39.1.(iv). Nevertheless, a search has been executed for these claims. The search has been based on the alleged effects of the compounds/compositions.
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

See extra sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☒ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

According to Article 34(3) (a-c) and Rule 13.2, an international application shall relate to one invention only or to a group of inventions linked by one or more of the same or corresponding "special technical features", i.e. features that define a contribution which each of the inventions makes over the prior art. All claims of the present application relate to alginate encapsulated cells producing tumour inhibitors. However, as this feature is not novel, see eg. WO-A1-9738707, the claims lack the "special technical feature" demanded by Rule 13.2 and the following inventions or groups of inventions were found:

1. Alginate encapsulated cells producing a tumour inhibiting substance, according to claims 1, 13 and 14 (partially)
2. Alginate encapsulated cells producing different tumour inhibitors, according to claims 2-5 and 14 (partially)
3. An alginate encapsulated hybridoma cell according to claim 6 and 14 (partially)
4. An alginate encapsulated cell in which the tumour inhibitor expression can be turned on or off, according to claims 7 and 14 (partially)
5. Cells encapsulated in various alginate compositions, according to claims 8-10 and 14 (partially), 18 (partially) and 19 (partially)
6. Microbeads of alginate encapsulated cells, according to claims 11 and 14 (partially)
7. Alginate encapsulated cells producing a brain tumour inhibitor, according to claims 12 and 14 (partially)
8. Implanting tumour inhibitor producing cells after surgical removal of a brain tumour, according to claim 15, 17, 18 (partially) and 19 (partially)

The characterising feature of invention 1 is the encapsulation of the cells in an immunoisolating alginate. This feature is known through the above mentioned WO-A1-9738707.

Invention 2 is characterised by the tumour inhibitors used.

Invention 3 is characterised by the cell type used.

Invention 4 is characterised by the possibility to turn the expression of tumour inhibitor on or off.

Invention 5 is characterised by the alginate compositions used.

Invention 6 is characterised by the physical form of the pharmaceutical product.

Invention 7 is characterised by the type of tumour targeted for inhibition.

Invention 8 is characterised by the method in which the encapsulated cells are used.

In summary, inventions 1-8 are not linked by a special technical feature as required by Rule 13.2 and the application therefore lacks unity of invention.

INTERNATIONAL SEARCH REPORT
Information on patent family members

02/11/99

International application No.
PCT/NO 99/00266

Patent document cited in search report.			Publication date	Patent family member(s)		Publication date
WO	9738707	A1	23/10/97	AU	2674097 A	07/11/97
WO	9628029	A1	19/09/96	AU	5186596 A	02/10/96
				CA	2215039 A	19/09/96
				EP	0813365 A	29/12/97
				JP	11501928 T	16/02/99
WO	9219195	A1	12/11/92	AT	156344 T	15/08/97
				AU	666118 B	01/02/96
				AU	682796 B	16/10/97
				AU	2004192 A	21/12/92
				AU	3902095 A	01/02/96
				DE	69221484 D,T	19/02/98
				DK	585368 T	16/03/98
				EP	0585368 A,B	09/03/94
				SE	0585368 T3	
				ES	2107537 T	01/12/97
				FI	934545 A	01/12/93
				GR	3025301 T	27/02/98
				HK	1001832 A	00/00/00
				JP	6507412 T	25/08/94
				NO	933842 A	25/10/93
				SG	47470 A	17/04/98
				US	5798113 A	25/08/98
				US	5800828 A	01/09/98
				US	5834001 A	10/11/98
				US	5869077 A	09/02/99
				US	5871767 A	16/02/99
				US	5874099 A	23/02/99